

EMBRYO IMPLANTATION: The role of αv family integrins

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PREFACE

CONTENTS

| | |
|---|----|
| Title page | 1 |
| Preface | 2 |
| Contents | 3 |
| List of Figures | 8 |
| List of Tables | 10 |
| Abstract | 11 |
| Declaration | 12 |
| Statement of copyright | 12 |
| Statement of ownership of intellectual property rights | 12 |
| Acknowledgments | 13 |
| Abbreviations | 14 |
| Chapter 1 Introduction | 16 |
| 1.1 GENERAL INTRODUCTION | 17 |
| 1.2 OVARIAN STEROID HORMONES | 18 |
| 1.2.1 Hormonal control of the endometrial cycle | 18 |
| 1.2.2 Steroidal control of early pregnancy in the mouse | 20 |
| 1.2.3 Steroid hormone receptors | 20 |
| 1.3 THE ENDOMETRIUM | 23 |
| 1.3.1 The endometrial cycle | 23 |
| 1.3.2 Uterine receptivity | 25 |
| 1.3.3 Decidualisation of the endometrial stroma | 29 |
| 1.4 EARLY EMBRYO DEVELOPMENT | 30 |
| 1.4.1 Fertilisation | 30 |
| 1.4.2 Pre-implantation embryo development | 31 |
| 1.5 IMPLANTATION | 35 |
| 1.5.1 Mechanisms of implantation | 35 |
| 1.5.2 In vitro models for implantation | 38 |
| 1.5.3 Placentation | 40 |
| 1.5.4 Local regulators of implantation | 40 |
| 1.6 BLASTOCYST ATTACHMENT | 42 |
| 1.6.1 Glycoconjugates | 42 |
| 1.6.2 Cell adhesion molecules | 45 |
| 1.7 INTEGRIN CELL ADHESION MOLECULES | 47 |
| 1.7.1 Integrin-ligand diversity | 49 |
| 1.7.2 Integrin-ligand binding | 52 |
| 1.7.3 The intracellular face of integrins | 55 |
| 1.7.4 'Outside-in' transduction of information through integrins | 57 |
| 1.7.5 Integrin-dependent signals converge with other cellular systems | 60 |
| 1.7.6 Integrins in implantation | 63 |
| 1.8 EXTRACELLULAR MATRIX COMPONENTS | 69 |

| | | |
|-----------|---|-----|
| 1.8.1 | Expression and function of ECM ligands in implantation | 71 |
| 1.8.2 | In vitro trophoblast outgrowth on ECM substrates | 73 |
| 1.9 | AIMS OF THE STUDY | 77 |
| Chapter 2 | Materials and methods | 78 |
| 2.1 | INTEGRIN EXPRESSION AND FUNCTION IN TISSUES | 79 |
| 2.1.1 | Human endometrium | 79 |
| 2.1.2 | Mouse endometrium collection | 79 |
| 2.1.3 | Immunocytochemistry of endometrial tissue | 80 |
| 2.1.4 | Human embryo acquisition | 80 |
| 2.1.5 | Immunocytochemistry of human blastocysts | 83 |
| 2.1.6 | Mouse embryo collection and culture | 85 |
| 2.1.6.1 | Superovulation | 85 |
| 2.1.6.2 | Oviduct flushing | 85 |
| 2.1.6.3 | Embryo culture | 85 |
| 2.1.7 | Immunocytochemistry of murine embryos | 86 |
| 2.1.8 | Mouse embryo outgrowth in vitro in serum-containing medium | 87 |
| 2.1.9 | Immunocytochemistry of mouse embryo outgrowths | 88 |
| 2.1.10 | Mouse embryo outgrowth in vitro in serum-free medium | 89 |
| 2.1.10.1 | Substrate coating | 89 |
| 2.1.10.2 | Serum-free outgrowth culture | 89 |
| 2.1.10.3 | Confirmation of thrombospondin coating | 89 |
| 2.1.11 | Addition of function blocking antibodies to outgrowth cultures | 90 |
| 2.1.11.1 | Determination of antibody dose required | 90 |
| 2.1.11.2 | Outgrowth analysis | 90 |
| 2.1.12 | Injection of an anti- α_v antibody into mouse uterus in vivo | 91 |
| 2.1.13 | Purification of an anti- α_v antibody from the C8F12 hybridoma clone | 91 |
| 2.1.13.1 | Maintenance of the C8F12 cell line | 91 |
| 2.1.13.2 | Purification of hybridoma supernatant | 92 |
| 2.1.14 | Confocal microscopy | 93 |
| 2.2 | MOLECULAR TECHNIQUES USED TO SUBCLONE $\beta 5$ cDNAs | 94 |
| 2.2.1 | Preparation of competent <i>Escherichia coli</i> (E. coli) | 94 |
| 2.2.2 | Transformation of E. coli strains MC1061 and TOP10F' | 94 |
| 2.2.3 | Preparation of purified plasmid DNA | 96 |
| 2.2.4 | Determination of DNA quantity and purity | 96 |
| 2.2.5 | Restriction enzyme digestion | 96 |
| 2.2.6 | Agarose gel electrophoresis of DNA | 97 |
| 2.2.7 | Purification of DNA fragments by gel slice elution | 97 |
| 2.2.8 | Oligonucleotide primer design | 97 |
| 2.2.9 | Polymerase chain reaction | 99 |
| 2.2.10 | DNA Ligation | 100 |
| 2.2.11 | Phosphatase treatment of pAcSecG2T plasmid DNA | 101 |
| 2.2.12 | Phenol-chloroform extraction | 101 |
| 2.2.13 | Ethanol precipitation | 102 |
| 2.2.14 | Automated DNA sequencing | 102 |
| 2.3 | PRODUCTION AND ANALYSIS OF RECOMBINANT PROTEINS | 103 |

| | | |
|-----------|---|-----|
| 2.3.1 | General handling and maintenance of the SF9 insect cell line | 103 |
| 2.3.1.1 | Checking cell viability | 103 |
| 2.3.1.2 | Adapting cells to protein-free medium | 103 |
| 2.3.1.3 | Adapting cells to suspension culture | 105 |
| 2.3.1.4 | Freezing and thawing cell stocks | 105 |
| 2.3.1.5 | Counting cells | 105 |
| 2.3.2 | Co-transfection of SF9 cells to produce recombinant baculovirus | 106 |
| 2.3.2.1 | Calcium phosphate co-precipitation | 106 |
| 2.3.2.2 | Lipofectin mediated co-transfection | 107 |
| 2.3.3 | Determination of virus titre | 107 |
| 2.3.3.1 | Setting up the plaque assay | 107 |
| 2.3.3.2 | Plaque identification and selection | 108 |
| 2.3.3.3 | Calculation of virus titre | 109 |
| 2.3.3.4 | End point dilution assay for assessment of virus titre | 109 |
| 2.3.4 | Amplification of virus titre from a plaque pick | 110 |
| 2.3.4.1 | Calculation of inoculum volume required | 110 |
| 2.3.4.2 | Amplification of virus titre | 110 |
| 2.3.5 | Examination of the SF9 genome for viral infection | 112 |
| 2.3.5.1 | Infection of cells with recombinant baculovirus | 112 |
| 2.3.5.2 | Extraction and analysis of genomic DNA | 112 |
| 2.3.6 | Small scale extraction of SF9 cell proteins using SDS | 112 |
| 2.3.7 | Extraction of SF9 cell proteins without SDS | 113 |
| 2.3.7.1 | Small scale extraction of SF9 cell proteins without SDS | 113 |
| 2.3.7.2 | Large scale extraction of SF9 cell proteins using detergent | 114 |
| 2.3.8 | Purification of fusion proteins using a glutathione affinity column | 115 |
| 2.3.8.1 | Preparation of glutathione Sepharose 4B | 115 |
| 2.3.8.2 | Binding of recombinant protein to glutathione column | 115 |
| 2.3.8.3 | Elution of GST from glutathione Sepharose 4B | 115 |
| 2.3.8.4 | GST detection assay | 116 |
| 2.3.9 | Determination of protein concentration | 117 |
| 2.3.9.1 | Lowry protein assay | 117 |
| 2.3.9.2 | Bradford protein assay | 117 |
| 2.3.10 | Polyacrylamide gel electrophoresis | 118 |
| 2.3.10.1 | Coomassie staining of gels | 121 |
| 2.3.10.2 | Silver staining of gels | 121 |
| 2.3.11 | Western blotting | 121 |
| 2.3.11.1 | Protein transfer | 121 |
| 2.3.11.2 | Antibody binding | 122 |
| 2.3.11.3 | Chemiluminescent detection | 122 |
| Chapter 3 | Expression and regulation of αv integrins in endometrium | 123 |
| 3.1 | INTRODUCTION | 124 |
| 3.2 | RESULTS | 124 |
| 3.2.1 | Preliminary investigations | 124 |
| 3.2.2 | Human endometrium | 125 |
| 3.2.2.1 | $\alpha v \beta 5$ integrin expression in human endometrium and decidua | 125 |
| 3.2.3 | Mouse endometrium | 128 |

| | | |
|---|--|-----|
| 3.2.3.1 | $\beta 5$ subunit expression | 128 |
| 3.2.3.2 | $\beta 3$ subunit expression | 131 |
| 3.2.3.3 | αv subunit expression | 134 |
| 3.3 | DISCUSSION | 137 |
| Chapter 4 | Expression of αv integrins in early embryos | 143 |
| 4.1 | INTRODUCTION | 144 |
| 4.2 | RESULTS | 144 |
| 4.2.1 | Human blastocysts | 144 |
| 4.2.1.1 | αv family integrin expression by human blastocysts | 147 |
| 4.2.2 | Pre- and peri-implantation stage mouse embryos | 147 |
| 4.2.2.1 | Development of mouse embryos in vitro | 148 |
| 4.2.2.2 | αv family integrin expression by mouse blastocysts | 150 |
| 4.2.3 | An in vitro model of mouse implantation | 153 |
| 4.2.3.1 | Development of trophoblast outgrowths in vitro | 153 |
| 4.2.3.2 | αv family integrin expression in trophoblast outgrowths | 155 |
| 4.3 | DISCUSSION | 161 |
| Chapter 5 | An investigation of integrin function in implantation | 165 |
| 5.1 | INTRODUCTION | 166 |
| 5.2 | RESULTS | 166 |
| Part 1 - Integrin function in an in vitro model of mouse implantation | | 166 |
| 5.2.1 | Optimisation of outgrowth cultures | 166 |
| 5.2.2 | Assessment and classification of outgrowth stage | 167 |
| 5.2.3 | Presentation and analysis of data from outgrowth studies | 168 |
| 5.2.4 | The ability of various substrates to support trophoblast outgrowth | 168 |
| 5.2.4.1 | The effect of substrate type on stage of outgrowth achieved | 168 |
| 5.2.4.2 | Confirmation of thrombospondin substrate coating | 173 |
| 5.2.5 | Production and purification of an αv function blocking antibody | 173 |
| 5.2.6 | Determination of antibody dose required to inhibit outgrowth | 175 |
| 5.2.7 | The αv and $\beta 1$ integrin subunits support embryo outgrowth | 175 |
| 5.2.7.1 | Vitronectin substrate | 176 |
| 5.2.7.2 | Fibronectin substrate | 181 |
| 5.2.7.3 | EHS matrix substrate | 186 |
| Part 2 - Investigation of integrin function in vivo: feasibility studies | | 191 |
| 5.2.8 | Setting up and assessment of the intra-uterine injection method | 191 |
| 5.2.8.1 | Intra-uterine injection of a function blocking anti- αv antibody | 192 |
| 5.3 | DISCUSSION | 194 |
| Chapter 6 | Production of $\beta 5$ integrin fusion proteins for immunisation | 202 |
| 6.1 | INTRODUCTION | 203 |
| 6.2 | RESULTS | 205 |
| Part 1 - Subcloning of $\beta 5$ DNA fragments into a baculovirus transfer vector | | 205 |
| 6.2.1 | Purification of the mouse $\beta 5$ integrin DNA fragment | 205 |

| | | |
|----------------|---|-----|
| 6.2.2 | Restriction enzyme site mapping of mouse $\beta 5$ integrin DNA | 205 |
| 6.2.3 | PCR amplification of full length and truncated $\beta 5$ DNA fragments for ligation into pAcSecG2T | 206 |
| 6.2.4 | Cloning of PCR products for verification of engineered Bam HI sites | 208 |
| 6.2.5 | Ligation of $\beta 5$ DNA fragments into the baculovirus transfer vector | 208 |
| 6.2.6 | Partial sequencing of plasmids pcDNA2-EXCYp & pcDNA2-EXTMp | 208 |
| Part 2 | - Production, selection and amplification of recombinant baculovirus | 210 |
| 6.2.7 | Co-transfection of SF9 cells to produce recombinant baculovirus | 210 |
| 6.2.7.1 | Calcium phosphate co-precipitation mediated co-transfection | 210 |
| 6.2.7.2 | Virus titre of calcium phosphate co-transfection supernatant | 210 |
| 6.2.7.3 | Lipofectin mediated co-transfection | 213 |
| 6.2.7.4 | Virus titre of Lipofectin mediated co-transfection supernatant | 213 |
| 6.2.8 | Selection and amplification of recombinant baculovirus clones | 216 |
| Part 3 | - Extraction of DNA and protein for analysis and purification | 218 |
| 6.2.9 | Morphological features of baculovirus-infected SF9 insect cells | 218 |
| 6.2.10 | Examination of recombinant baculovirus-infected SF9 cell genomic DNA for sequence encoding mouse $\beta 5$ integrin subunit | 218 |
| 6.2.11 | Analysis of proteins produced by SF9 cell cultures | 221 |
| 6.2.11.1 | Following the time course of fusion protein production | 221 |
| 6.2.11.2 | Confirmation that extracted fusion protein is $\beta 5$ integrin | 221 |
| 6.2.11.3 | Secretion of fusion proteins into culture medium by SF9 cells | 224 |
| 6.2.11.4 | Extraction of protein from SF9 cells without denaturation | 226 |
| 6.2.11.5 | Large scale protein production and purification | 228 |
| 6.2.11.6 | Immunisation of $\beta 5$ fusion protein | 231 |
| 6.3 | DISCUSSION | 232 |
| Chapter 7 | General discussion | 241 |
| References | | 251 |
| Appendices | | 286 |
| Appendix 1 | Immunocytochemistry reagents | 287 |
| Appendix 2 | Molecular biology solutions | 288 |
| Appendix 3 | Biochemistry solutions | 289 |
| Appendix 4 | Outgrowth culture raw data and control statistics | 290 |
| Appendix 5 | Restriction enzyme map of known mouse $\beta 5$ sequence | 294 |
| Appendix 6 | Negative control human endometrium and decidua | 297 |
| Published work | | 298 |

LIST OF FIGURES

| | | |
|-------------|---|-----|
| Figure 1.1 | Circulating hormone levels in women and rodents | 19 |
| Figure 1.2 | Early embryo development in the mouse | 32 |
| Figure 1.3 | A summary of integrin subunit structure | 48 |
| Figure 1.4 | Structural features of extracellular matrix components | 70 |
| Figure 2.1 | Apparatus used in embryo studies | 84 |
| Figure 2.2 | Alignment of partial mouse $\beta 5$ integrin cDNA against human | 98 |
| Figure 3.1 | Immunolocalisation of αv and $\beta 5$ integrins in human endometrium | 126 |
| Figure 3.2 | Immunolocalisation of $\beta 5$ integrins in mouse endometrium | 129 |
| Figure 3.3 | Immunolocalisation of $\beta 3$ integrins in mouse endometrium | 132 |
| Figure 3.4 | Immunolocalisation of αv integrins in mouse endometrium | 135 |
| Figure 4.1 | Immunolocalisation of αv integrins in human blastocysts | 145 |
| Figure 4.2 | Development of mouse embryos in vitro | 149 |
| Figure 4.3 | Immunolocalisation of αv integrins in mouse embryos | 151 |
| Figure 4.4 | Immunolocalisation of $\beta 5$ integrins in a mouse blastocyst | 152 |
| Figure 4.5 | Development of trophoblast outgrowths in vitro | 154 |
| Figure 4.6 | Immunolocalisation of αv integrins in trophoblast outgrowths | 156 |
| Figure 4.7 | Immunolocalisation of $\beta 1$ integrins in trophoblast outgrowths | 157 |
| Figure 4.8 | Immunolocalisation of $\beta 3$ integrins in trophoblast outgrowths | 158 |
| Figure 4.9 | Immunolocalisation of $\beta 5$ integrins in trophoblast outgrowths | 159 |
| Figure 5.1 | Effect of substrate on trophoblast outgrowth development | 170 |
| Figure 5.2 | Effect of substrate on proportion of embryos outgrowing | 171 |
| Figure 5.3 | Immunodetection of thrombospondin to confirm coating | 174 |
| Figure 5.4 | Anti- αv antibody purified from C8F12 hybridoma medium | 174 |
| Figure 5.5 | Trophoblast outgrowth on vitronectin without serum | 177 |
| Figure 5.6 | Trophoblast outgrowth development on vitronectin | 178 |
| Figure 5.7 | Effect of integrin inhibition on outgrowth on vitronectin | 179 |
| Figure 5.8 | Trophoblast outgrowth on fibronectin without serum | 182 |
| Figure 5.9 | Trophoblast outgrowth development on fibronectin | 183 |
| Figure 5.10 | Effect of integrin inhibition on outgrowth on fibronectin | 184 |
| Figure 5.11 | Trophoblast outgrowth on EHS matrix without serum | 187 |
| Figure 5.12 | Trophoblast outgrowth development on EHS matrix | 188 |
| Figure 5.13 | Effect of integrin inhibition on outgrowth on EHS matrix | 189 |
| Figure 6.1 | A summary of the insect cell baculovirus expression system | 204 |
| Figure 6.2 | Molecular analysis, amplification and subcloning of $\beta 5$ cDNA | 207 |
| Figure 6.3 | Partial sequencing of $\beta 5$ -plasmids aligned against human $\beta 5$ | 209 |
| Figure 6.4 | Co-transfection of SF9 cells by calcium phosphate co-precipitation | 212 |
| Figure 6.5 | Co-transfection of SF9 cells using Lipofectin | 214 |
| Figure 6.6 | Estimation of virus titre by plaque assay | 217 |

| | | |
|-------------|---|-----|
| Figure 6.7 | Morphological and genetic evidence of recombinant virus Infection | 219 |
| Figure 6.8 | A study of the time course of fusion protein production | 222 |
| Figure 6.9 | Identification of fusion protein as mouse $\beta 5$ integrin | 223 |
| Figure 6.10 | Detection of fusion protein in SF9 cell-conditioned medium | 225 |
| Figure 6.11 | Protein extraction from SF9 cells using different lysis buffers | 227 |
| Figure 6.12 | Large-scale production and purification of fusion protein | 229 |
| Figure 7.1 | Embryo implantation: a model for the role of αv integrins | 250 |

LIST OF TABLES

| | | |
|--------------|--|-----|
| Table 1.1 | Uterine changes during the estrus cycle | 24 |
| Table 1.2 | Mechanisms for producing apical cell surface adhesiveness | 26 |
| Table 1.3 | Changes in uterine epithelium with acquisition of 'receptivity' | 28 |
| Table 1.4 | Alternative mechanisms of trophoblast invasion | 37 |
| Table 1.5 | Extracellular matrix ligands of $\beta 1$ - and αv -family integrins | 50 |
| Table 1.6 | Steps involved in integrin-dependent signal transduction | 58 |
| Table 2.1 | Primary antibodies | 81 |
| Table 2.2 | Secondary antibodies | 82 |
| Table 2.3 | Key to plasmid terminology | 95 |
| Table 2.4 | Culture of SF9 cells | 104 |
| Table 2.5 | Conditions for virus amplifications from plaque picks | 111 |
| Table 2.6 | Preparation of polyacrylamide gels | 119 |
| Table 2.7 | Protein standard markers | 120 |
| Table 3.1 | $\alpha v \beta 5$ expression in human endometrium and decidua | 127 |
| Table 3.2 | $\beta 5$ integrin subunit expression in mouse endometrium | 130 |
| Table 3.3 | $\beta 3$ integrin subunit expression in mouse endometrium | 133 |
| Table 3.4 | αv integrin subunit expression in mouse endometrium | 136 |
| Table 4.1 | Integrin subunit expression by human blastocysts | 146 |
| Table 4.2 | αv family integrin expression in trophoblast outgrowths | 160 |
| Table 5.1 | Successful embryo outgrowth - significance of substrate type | 172 |
| Table 5.2 | Outgrowth on vitronectin - significance of culture condition | 180 |
| Table 5.3 | Outgrowth on fibronectin - significance of culture condition | 185 |
| Table 5.4 | Outgrowth on EHS matrix - significance of culture condition | 190 |
| Table 5.5 | Effect of intra-uterine injections on implantation rate | 193 |
| Table 6.1 | Calculation of virus titre by plaque assay | 215 |
| Table 6.2 | Morphological signs of baculovirus infection of SF9 cells | 220 |
| Table 6.3 | Spectrophotometric assay of relative GST activity of eluates | 230 |
| Appendix 4.1 | Stage of outgrowth achieved with differing culture conditions | 290 |
| Appendix 4.2 | Effect of antibody concentration on embryo outgrowth inhibition | 291 |
| Appendix 4.3 | Substrate dependent effects of culture condition | 292 |
| Appendix 4.4 | Effect of culture condition compared to negative control substrate | 293 |

ABSTRACT

Implantation requires direct adhesion of embryonic trophectoderm to the luminal epithelium of the endometrium, with subsequent penetration of the uterine stroma. This process is dependent on endometrial differentiation and embryo development, and culminates in a cascade of cell adhesion events. This study aimed to examine the role of integrins - a large family of heterodimeric cell adhesion molecules which initiate intracellular signalling cascades. A subgroup of integrins that share a common α subunit (α_v) was the focus of the study. Wherever possible, comparison of implantation in human and mouse was made.

The expression of α_v integrins in human and mouse endometrium around the time of implantation was demonstrated (Aplin et al, 1996). The α_v , β_3 and β_5 subunits showed remarkable localisation to the apical epithelium, the surface at which attachment occurs. The basal surface of the luminal epithelium stained intensely for β_5 (but not α_v) in mouse, suggesting the possible presence of an as yet unidentified α subunit. Endometrial integrins did not appear to be steroidally regulated. Human blastocyst trophectoderm cells express α_v , β_1 , β_3 and β_5 ; these were also demonstrated in mouse blastocysts. These data demonstrated that α_v integrins are available to mediate embryo attachment.

Using an in vitro model of mouse implantation in which trophoblast cells form outgrowths on tissue culture glass coated with serum proteins, differential localisation of the α_v , β_1 , β_3 and β_5 subunits was observed. This suggested several functions during trophoblast invasion in utero. α_v and β_3 subunits appeared in focal adhesions on the lower (substrate) surface of trophoblasts, while β_1 and β_5 were seen in large clusters on the upper surface of these cells; this provided further evidence for an alternative partner for β_5 in the mouse. In a modification of this model, outgrowths were maintained in the absence of serum on defined substrates. By addition of integrin function-blocking antibodies to these cultures it was shown that α_v was critical for trophoblast outgrowth on fibronectin or vitronectin, while outgrowth on laminin required β_1 . The possibility of blocking mouse implantation in utero was investigated, but experimental difficulties prevented a direct approach to evaluation of integrin function.

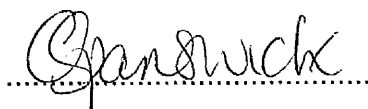
To further dissect the roles of specific α_v integrin pairs in implantation, function-blocking antibodies specific to individual mouse β subunits are required. As these were of limited availability, a fusion protein of mouse β_5 was produced using the insect cell baculovirus expression system. The extracellular domain of mouse β_5 was extracted, purified and subsequently used for immunisation. The resultant polyclonal antisera are currently being characterised in our laboratory.

This study highlighted several potential roles for α_v integrins in implantation, and that these may be common to both mouse and human. During embryo attachment, α_v integrins on the maternal and embryonic surfaces may bind a shared bridging ligand. With trophoblast invasion, several α_v integrin-ligand interactions may be important, culminating in intracellular signalling events.

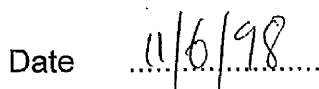
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ABBREVIATIONS

| | | | |
|------------------|-------------------------------------|-------------|--------------------------------|
| $\alpha(1-2)$ FT | $\alpha(1-2)$ fucosyl transferase | EGF-R | EGF receptor |
| AA | amino acid | EHS | Engelbreth-Holm-Swarm |
| AcMNPV | <i>Autographa californica</i> | EN | entactin |
| | multiple nuclear polyhedrosis virus | ER | oestrogen receptor |
| ADAM | a disintegrin and | ERKO | ER knockout |
| | metalloproteinase domain | EXCY | full length $\beta 5$ |
| APS | ammonium persulphate | EXTM | $\beta 5$ ectodomain |
| AR | amphiregulin | FAK | focal adhesion kinase |
| BSA | bovine serum albumin | FB | fibrinogen |
| CAM | cell adhesion molecule | FCS | fetal calf serum |
| CIP | calf intestinal phosphatase | FGF | fibroblast growth factor |
| CNDB | 1-chloro-2,4-dinitrobenzene | FITC | fluorescein isothiocyanate |
| COL | collagen | FN | fibronectin |
| CSF-1 | colony stimualting factor-1 | FSH | follicle stimualting hormone |
| CSPG | chondroitin suphate | G-CSF | granulocyte CSF |
| | proteoglycan | GST | glutathione-S-transferase |
| CTB | cytotrophoblast | HB-EGF(-TM) | heparin binding EGF |
| DABCO | 1, 4 diazobicyclo-(2, 2, 2) | | (transmembrane form) |
| | octane | HBSS | Hank's balanced salt solution |
| DDT | dithiothreitol | hCG | human chorionic |
| DMEM | Dulbeccos modified Eagles | | gonadotrophin |
| | medium | HEPES | 4-(2-hydroxyethyl-1- |
| DMSO | dimethyl sulphoxide | | piperazineethanesulphonic acid |
| DNA | deoxyribonucleic acid | HIP | heparan sulphate interacting |
| E ¹ | oestrogen priming | | protein |
| E ² | nidatory oestrogen | HSPG | heparan sulphate |
| E. coli | <i>Escherichia coli</i> | | proteoglycan |
| ECM | extracellular matrix | IAP | integrin associated protein |
| EDTA | ethylenediaminetetraacetic | ICM | inner cell mass |
| | acid | IGF | insulin-like growth factor |
| EGF | epidermal growth factor | IGF-BP1 | IGF binding protein-1 |

| | | | |
|-----------------|---|---------|--|
| IGF-R | IGF receptor | PDGF | platelet derived growth factor |
| IgG | immunoglobulin G | PECAM-1 | platelet endothelial CAM |
| IgSF | Ig superfamily | PFA | paraformaldehyde |
| IL | interleukin | PFU | plaque forming units |
| IL-1R | IL-1 receptor | PKC | protein kinase C |
| ILK | integrin linked kinase | PMSF | α -toluenesulfonyl fluoride |
| IPTG | X-Gal-isopropylthio- β -D-galactoside | PR | progesterone receptor |
| IVF | in vitro fertilisation | SDS | sodium dodecyl sulphate |
| LB | Lauria Bertini | SF | <i>Spodoptera frugiperda</i> |
| Le ^y | Lewis ^y epitope | STE | sodium Tris EDTA |
| LH | lutensising hormone | TAE | Tris acetate EDTA |
| LIBS | ligand induced binding site | TE | Tris EDTA |
| LIF | lukaemia inhibitory factor | TEMED | N, N, N, N, -tetra-methyl-ethylenediamine |
| LM | laminin | TGF | transforming growth factor |
| LNF-1 | lacto-N-fucopentaose | TN | tenascin |
| M-CSF | macrophage CSF | TNF | tumour necrosis factor |
| MAdCAM | mucosal addressin CAM | TNF-R | TNF receptor |
| MAPK | mitogen activated protein kinase | TSP | thrombospondin |
| MMP-2 | matrix metalloproteinase-2 | TX-A | Triton-X 100 lysis buffer A |
| MOI | multiplicity of infection | TX-B | Triton-X 100 lysis buffer B |
| mRNA | messenger ribonucleic acid | uPAR | urokinase type plasminogen activating receptor |
| N-CAM | neuronal CAM | VCAM | vascular CAM |
| OST | osteopontin | VEGF | vascular endothelial growth factor |
| P | progesterone | VN | vitronectin |
| PAGE | polyacrylamide gel electrophoresis | vs. | versus |
| PAI-1 | plasminogen activating inhibitor | vWF | von Willebrand factor |
| PBS | phosphate buffered saline | ZO-1 | zona occludin |
| PCR | polymerase chain reaction | ZP | zona pellucida |

CHAPTER 1

Introduction

1.1 GENERAL INTRODUCTION

Implantation is a fundamental mechanism of mammalian biology whereby an embryo establishes stable contact with the uterus of its mother. For an embryo to implant into the uterus it must have developed to the blastocyst stage, and be surrounded by the first differentiated cell type - the trophectoderm epithelium. The endometrium (lining of the uterus) undergoes regular cyclical changes controlled by ovarian steroid hormones. Only at a specific stage during the endometrial cycle is the uterus 'receptive' to a blastocyst. At implantation it is the trophectoderm which comes into first contact with the surface epithelium of the uterus. A common feature of virtually all epithelia is that their apical surface is non-adhesive. Therefore, the adhesion of the trophectoderm and endometrial epithelia via their respective apical surfaces at implantation represents an extraordinary phenomenon in cell biology.

Embryo attachment is thought to involve a cascade of adhesive events, the precise nature of which are unknown. In species in which implantation is invasive, initial attachment is followed by penetration of endometrial epithelium and underlying stroma. Trophoblast invasion is precisely regulated and requires further adhesive interactions. It is of great biological importance that we can understand these critical early stages of implantation, and of clinical significance to infertility and other diseases of the endometrium and pregnancy.

For physiological and ethical reasons it is difficult to study implantation in humans, therefore many studies focus on laboratory animals and use in vitro models of implantation. Such studies demonstrate many similarities between implantation in human and rodents, and also show the value of in vitro studies. These models are of course not perfect, and care must be taken when extrapolating from these systems to human implantation. Nevertheless they have contributed greatly to, and will continue to provide much of, our understanding of reproductive biology.

1.2 OVARIAN STEROID HORMONES

1.2.1 Hormonal control of the endometrial cycle

In human and mouse the endometrium undergoes regular cyclical changes (section 1.3.1) that are directly controlled by a predictable pattern of steroid hormones from the ovary (Figure 1.1). In women this is termed the menstrual cycle and typically lasts 28 days. In rodents similar although less extreme changes occur over a period of 4-5 days known as the estrus cycle.

Secretion of ovarian steroids is strictly controlled by changing levels of follicle stimulating hormone (FSH) and lutenising hormone (LH; Figure 1.1). These are produced by the anterior pituitary under the control of hypothalamic gonadotrophin-releasing hormones. FSH and LH control the development and maturation of oocytes in preparation for ovulation. During the ovarian 'follicular' phase (endometrial 'proliferative' phase) several oocytes begin development. FSH stimulates oestrogen secretion by these developing follicles. With rising oestrogen levels, a mid-cycle surge in LH (and FSH) induces rupture of the dominant follicle, resulting in ovulation. Following oocyte release, the ovarian 'luteal' phase (endometrial 'secretory' phase) ensues, involving maturation of the corpus luteum which is required for subsequent progesterone production under the action of LH. Also of great significance is the requirement for oestrogen to produce the behavioral changes in female animals necessary for mating to occur.

Therefore, oestrogen dominates the first proliferative changes of the endometrial cycle. The effects of progesterone are evident in oestrogen-primed tissue (Martin & Finn, 1968). Rising levels of progesterone (with maintained oestrogen) are required for secretory differentiation of the endometrium in preparation for uterine receptivity. In rodents, progesterone-induced differentiation is essential for response to 'nidatory' oestrogen - a precisely timed surge of oestrogen critical for implantation in rodents and without which the uterus enters a non-receptive state (Finn & Martin, 1967; Psychoyos, 1986).

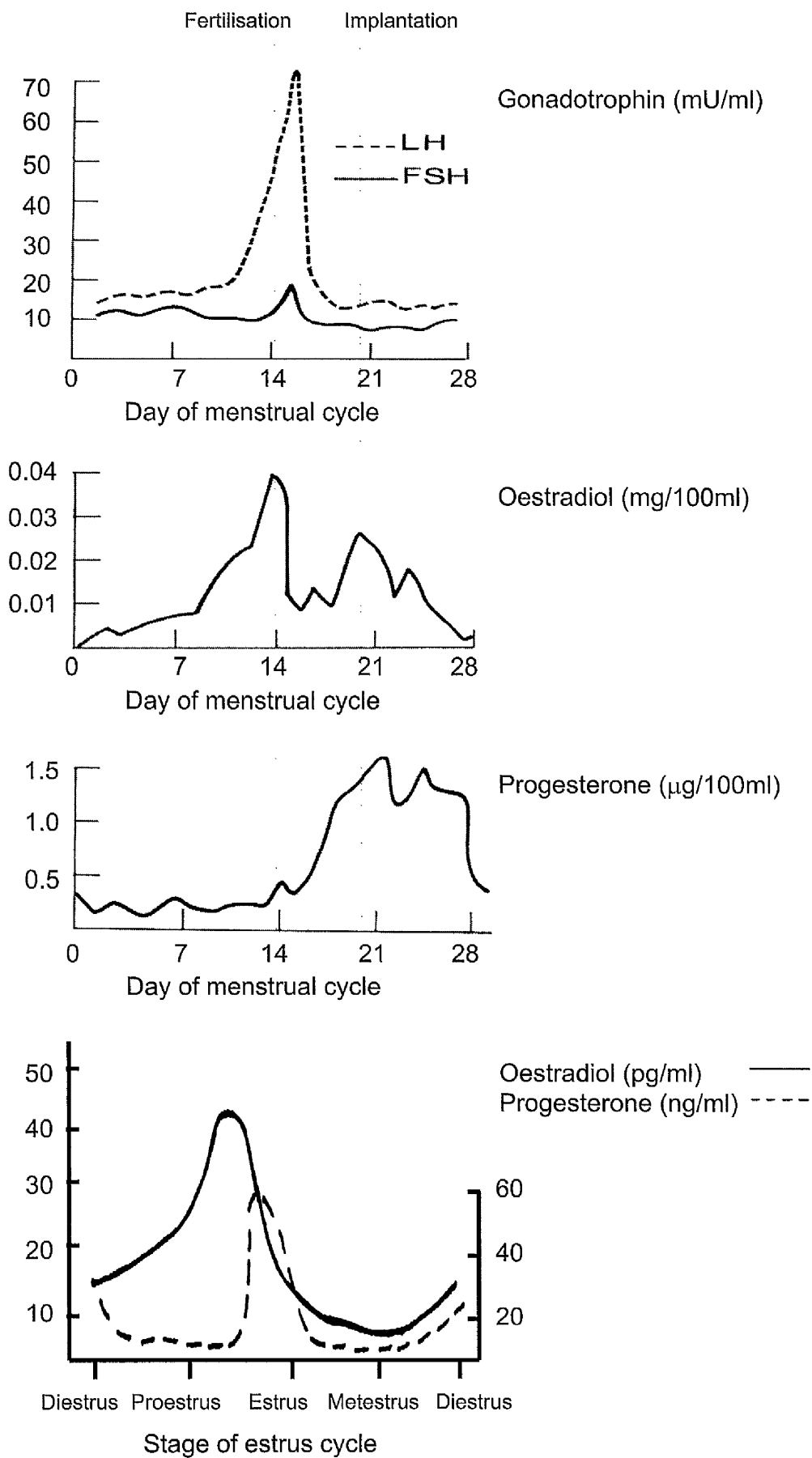


Figure 1.1 Circulating hormone levels in women and rodents

Adapted from Symonds, 1992.

1.2.2 Steroidal control of early pregnancy in the mouse

If mating is successful specific steroidal changes are required initially for implantation to occur, and then for pregnancy to be maintained. On day 1 of pregnancy oestrogen levels are high and then greatly reduced on days 2 and 3. Conversely, progesterone secretion is not seen until 48 hours post-coitum (Finn & Martin, 1969) with the development of the corpus luteum. The nidatory oestrogen surge occurs on day 4 of pregnancy and is essential for impending implantation (Finn & Martin, 1967; Psychoyos, 1986). To complement this, mouse blastocysts express oestrogen receptors in all cells (Hou *et al.*, 1996). Progesterone continues to rise, reaching a peak on day 6 of pregnancy, with a transient decrease on day 7 when hormonal control is switched from the pituitary to placenta. Progesterone levels remain high until approximately 4 days before birth, when oestrogen levels rise again (McCormack & Greenwald, 1974).

1.2.3 Steroid hormone receptors

The effect of ovarian hormones is dependent upon the expression of their respective receptors, all members of the nuclear receptor superfamily of transcription factors (Beato, 1989). The expression of the receptors for oestrogen (ER) and progesterone (PR) is strictly controlled by the secretion of the two sex steroids. Oestrogen increases the expression of ER and PR; conversely, under the influence of progesterone, levels of the two receptors are dramatically reduced (Bouchard *et al.*, 1991; McDonnell & Goldman, 1994). Progesterone downregulates occupied nuclear ER by stimulating ER turnover and inhibiting synthesis. This rapid effect is seen within 2 hours of progesterone treatment and seems to be via a factor which blocks ER acceptor sites, thereby stimulating turnover (Leavitt *et al.*, 1987). Hormone replacement regimes in women with ovarian failure produce the expected endometrial changes including a predictable pattern of expression of ER and PR (Massai *et al.*, 1993)

In human endometrium ER and PR are differentially expressed between the epithelial, stromal and vascular cell populations. Expression of ER is highest in proliferative endometrium, decreasing gradually through the post-ovulatory

secretory phase in both epithelial and, more markedly, stromal cells (Bergeron *et al.*, 1988). Levels of PR are highest in proliferative and early secretory phase endometria in stromal and, to a greater extent, epithelial cells. Dramatic disappearance of PR is then seen and continues throughout the mid-late secretory phase in the epithelia, whereas low levels of PR are maintained in stromal cells (Press *et al.*, 1988). Uterine arteries express ER and PR, where expression is restricted to arterial muscle cells, and absent from endothelial cells and uterine veins (Perrot-Applanat *et al.*, 1988). The distribution pattern of ER and PR in the uterus and Fallopian tube shows a gradient of positively stained cells, greatest in the upper part of the uterus (fundus) where implantation is known to preferentially occur (Coppens *et al.*, 1993).

In 1993, Lubahn *et al.* achieved the first known disruption of ER function by producing the ER-knockout mouse (ERKO). Cross-breeding of mice heterozygous for the ER null mutation followed typical Mendelian inheritance, where homozygotes were viable and of balanced male:female ratio. Disruption of the ER was not lethal; prenatal reproductive tract development appeared normal, but all ERKO mice born were infertile. Females had reproductive tracts but the uterus was unable to respond to oestrogen treatment, similar to the effect of ovariectomy in wild-type animals. The ovaries were haemocystic with no detectable corpora lutea, indicative of hyperstimulation by gonadotrophins due to disrupted negative feedback. Male ERKO mice had anatomically normal accessory sex organs but the testes were reduced in size with abnormal seminiferous tubules. Few germ cells or sperm were apparent (Lubahn *et al.*, 1993; Korach *et al.*, 1996). Smith *et al.* (1994) described a human adult male who presented with bone deformities (incomplete epiphyseal closure). He was identified as having a homozygous mutation of ER gene and he showed no response to exogenous oestrogen therapy. Recently a novel ER isoform has been identified (termed ER- β), which has a low affinity for oestradiol compared to the original ER (ER- α). Although ER- β shares many of the functional characteristics of ER- α , the mechanisms regulating its transcriptional activity

appear to be distinct (Tremblay *et al.*, 1997). It will be interesting to see whether ER- β knockouts shed any further light on the role of oestrogen in reproduction.

A significant positive correlation between PR expression in proliferative endometrium and circulating 17 β -oestradiol levels has been observed (Kreitmann *et al.*, 1979), but this is not extended to secretory phase endometrium. ER occupancy produces transcription of the PR gene 3 hours following oestrogen-receptor binding and leads to accumulation of PR mRNA 6 hours after oestrogen treatment (Lee & Gorski, 1996). This gradual effect of oestrogen on PR production does not parallel the time scale of oestrogen-receptor binding, which is maximal within 30 minutes of oestrogen treatment and greatly reduced by 3 hours. Therefore it appears that although ER ligation is essential for PR upregulation, mediating factors are likely to implement this effect. Two forms of human PR (hPR-A, hPR-B) are produced from a single gene. Induction of transcription by both ER and PR can be inhibited by hPR-A. Transcription of progesterone-responsive genes is activated by hPR-B. These effects are mediated by distinct structural regions of hPR-A/B (Wen *et al.*, 1994). PR knockout mice have been produced and survive to adulthood. Females are infertile, unable to exhibit sexual behavior (lordosis) and have several reproductive tract defects. Following combined oestrogen and progesterone treatment, the uterine epithelium of these mice become hyperplastic, as if oestrogen alone had been administered (Lydon *et al.*, 1995; 1996).

Early experiments providing steroid replacement to ovariectomised mice are central to our understanding of the steroidal control of endometrial differentiation and receptivity. More recently, by examining the effect of disrupted steroid receptor function, a great deal more has been learnt about reproductive development and function. Clearly, regulation of the endometrium by ovarian steroids is central to uterine receptivity and implantation, and these are likely to be mediated by a multitude of local tissue factors (section 1.5.4).

1.3 THE ENDOMETRIUM

Although there are species differences in endometrial ultrastructure and the time scale of the endometrial cycle, the central function of this tissue in all mammals is to support embryo development. The endometrium goes through extensive differentiative changes every cycle in anticipation that pregnancy may occur. There are three main functions of this process: (i) modification of uterine lumen milieu to support blastocyst growth and differentiation by altered glandular secretions (1.3.1); (ii) alteration of the luminal epithelium so that it becomes receptive to blastocyst attachment (1.3.2); (iii) decidualisation of the stroma (1.3.3), with accompanying vascular changes. Whether these changes rely on the presence of a blastocyst within the uterine cavity remain unclear.

1.3.1 The endometrial cycle

The cellular changes seen during the endometrial cycle are regulated by steroid hormones secreted by the ovary (1.2.1). In the human, during a typical 28 day cycle, the endometrium is transformed from a thin layer of proliferative epithelial and stromal cells (basalis) to a thick tissue full of coiled blood vessels and secretory glands (functionalis; Noyes *et al.*, 1950; Li *et al.*, 1994). This transformation occurs in preparation for receiving and nourishing an embryo. If fertilisation does not occur the blood vessels are constricted, and the functionalis is shed through menstruation. During the 4-5 day rodent estrus cycle the uterus undergoes similar cellular changes over four stages, summarised in Table 1.1.

In the mouse, oestrogen early in the endometrial cycle leads to increased mitosis in the luminal and glandular endometrial epithelium without similar effects on stromal cells (Finn & Martin, 1970). Protein synthesis, by the uterine epithelial, stromal and myometrial cell populations, is differentially regulated by oestrogen (Quarmby & Korach, 1984). Following oestrogen priming progesterone switches mitosis from epithelial cells to the stroma and is required to sensitise the uterus to the subsequent nidatory oestrogen surge (Martin & Finn, 1968). This nidatory oestrogen is required to continue the initial burst of stromal mitosis and to transform the stroma into decidua (1.3.3; Finn & Martin, 1969).

Table 1.1 Uterine changes during the estrus cycle

Proestrus

With rising oestrogen, the uterus swells due to stromal proliferation and oedema. Uterine epithelium begins mitosis and myometrium is contractile.

Estrus

As oestrogen levels peak epithelial mitosis is at its maximum and the uterus becomes highly distended.

Metestrus

Early metestrus: With rising progesterone, stromal proliferation increases.

Late metestrus: A peak in progesterone accompanied by the 'nidatory' oestrogen surge induces endometrial glands to release secretory material.

Diestrus

As hormone levels decline degeneration of the endometrium begins.

Adapted from Snell & Stevens, 1966.

1.3.2 Uterine receptivity

There exists a specific phase during the cycle when the endometrium is 'receptive' to implantation of a blastocyst (Yaron *et al.*, 1994). This was first demonstrated in rats, shown to be dependent on an oestrogen surge occurring on day 4 of pregnancy, where implantation occurs at approximately day 5.5 (Psychoyos, 1976; Psychoyos, 1986). If nidatory oestrogen is removed by ovariectomy early on day 4 of pregnancy, implantation does not occur. Here, the embryo is arrested at the blastocyst stage but will implant normally if transferred to an intact pseudopregnant mouse (Paria *et al.*, 1993b). In rodents it seems that the 'window of receptivity' lasts less than 24 hours, occurring from day 4.5 of pregnancy (Psychoyos, 1976). In women much less precise estimates regarding the length of the receptive period are possible, but the outcome of embryo transfer regimes suggest that implantation can occur from days 20 to 24 of the menstrual cycle (Bergh & Navot, 1992).

If the uterine epithelium is removed, blastocysts can implant into the uterus completely irrespective of its hormonal status (Cowell, 1969), but when intact the uterine epithelium can act as a barrier against implantation. Therefore, the uterine epithelium appears to play a pivotal role in regulating endometrial 'receptivity' (Denker, 1990). Although some species require a copulatory stimulus to induce uterine receptivity, in most mammals (including human and mouse) the regular cyclic changes in the endometrium that occur with every ovulation switch the uterus from a non-receptive to receptive state. This epithelial switch is in part mediated by signals from the underlying stroma, and also maintained by embryonic signals and stimulated further by local blastocyst-derived factors (Paria *et al.*, 1993b; Shiotani *et al.*, 1993). Outside the receptive period it seems that the uterine epithelium (and trophoblast) behave like typical epithelia, with non-adhesive apical surfaces. During the peri-implantation period the adhesive properties of the uterine epithelium apical surface change (Table 1.2).

Table 1.2 Mechanisms for producing apical cell surface adhesiveness

1. Loss of adhesion-inhibiting molecules from the apical cell surface
 2. Apical expression of newly synthesised cell adhesion components
 3. Coordinated synthesis of cell adhesion components with down-regulation of anti-adhesive molecules
 4. Re-distribution of existing cell adhesion components from the baso-lateral to the apical cell membrane
-

De-polarisation of the uterine epithelium occurs with receptivity

In a non-receptive state, the uterine epithelium is a typical columnar epithelium, being highly polarised with distinct apical, lateral and basal surfaces. This specialised epithelium provides a barrier protecting the endometrium from the external environment of the uterine lumen. Mouse uterine epithelial cells in vitro, when maintained in a polarised state, retain hormonal responsiveness (Glasser *et al.*, 1988; Glasser & Mulholland, 1993). Apico-basal polarity provides discrete functional domains within the cell, allowing processes such as secretion and ion transport to occur.

The altered phenotype of the uterine epithelium seen with the acquisition of receptivity is not restricted to the apical cell surface (Denker, 1990), as described in Table 1.3.. The parameters noted are typical features of epithelial cell apico-basal polarity, and the changes seen upon receptivity are suggestive of reduced polarity, phenotypic of a mesenchymal state (Carson *et al.*, 1990; Denker, 1990b, 1994; Thie *et al.*, 1996, 1995). Thus, there is scope for the uterine epithelium to modulate implantation.

The adhesive changes occurring with receptivity at the apical uterine epithelial cell surface are the subject of intense investigation. Various potential mechanisms for cell-cell attachment, including integrin-extracellular matrix interactions and carbohydrate mediated events have been identified. The possible role of these cell adhesion systems in blastocyst attachment at implantation is examined further in sections 1.6, 1.7 and 1.8.

Table 1.3 Changes in uterine epithelium with acquisition of 'receptivity'

-
1. Apical cell membrane
 Changes in composition and adhesive function:
 - Thickness of glycocalyx is reduced
 - Reduced cell surface negative charge
 - Reduced sialylation of surface glycoconjugates
 - Expression of new adhesion-mediating molecules
 2. Lateral cell membrane
 Junctional complexes no longer restricted to apico-lateral region:
 - Tight junctions increase and spread towards baso-lateral aspect of cell
 - Desmosomes diffuse along lateral surface (rabbits)
 3. Basal cell membrane
 Altered association with basal lamina:
 - Cell attachment to basal lamina reduced (rodents)
 - Cytoplasmic projections penetrate basal lamina (human, rabbit)
 4. Cytoskeleton
 Changes in vimentin:
 - Vimentin expression increases
 - Vimentin re-distributed from apical to basal compartment of cell

Morris & Potter, 1990^{1a}; Hewitt *et al.*, 1979^{1b}; Chavez, 1990^{1c}; Carson *et al.*, 1990a^{1d}; Murphy *et al.*, 1982^{2a}; Classen-Linke & Denker, 1990^{2b}; Tachi *et al.*, 1970^{3a}; Roberts *et al.*, 1988^{3b}; Hochfeld *et al.*, 1990⁴.

1.3.3 Decidualisation of the endometrial stroma

During the attachment stage of implantation the endometrial stroma is transformed from a spindle-shaped connective tissue to tightly packed, polyhedral 'decidual' cells. These accumulate intermediate filaments, glycogen and lipids, and establish intercellular junctions (Abrahamsohn & Zorn, 1993; Aplin & Glasser, 1994). In rodents, mitosis switches from the epithelial to stromal compartments under progesterone action (sections 1.2.1, 1.3.1; Martin & Finn, 1968). Nidatory oestrogen is required to continue this mitosis, and is involved in transforming the stroma into decidua (section 1.2.2; Finn & Martin, 1969). Decidualising stroma becomes highly active in a wave that spreads from the attachment site, synthesising macromolecules and secreting them into the extracellular matrix (ECM; section 1.8.1). Endothelial proliferation in the endometrium is also seen prior to implantation and is regulated by vascular endothelial growth factor (VEGF). In rodents VEGF increases vascular permeability at the site of blastocyst attachment and initiates angiogenesis in the decidualising stroma (Chakraborty *et al.*, 1995).

Decidualisation of the human endometrial stroma becomes apparent during the secretory phase of the menstrual cycle, under the influence of progesterone. Stromal cells surrounding endometrial blood vessels enlarge and differentiate and with pregnancy similar changes spread throughout the stroma (Tang *et al.*, 1994). Transformation of the endometrial stroma produces decidual cells that express an array of bioactive proteins such as cytokines (section 1.5.4), hormones and ECM components (section 1.8.1). Gradients of these molecules within the endometrium produce an environment that supports and controls trophoblast invasion during placentation (section 1.5.3; Abrahamsohn & Zorn, 1993; Aplin & Glasser, 1994; Simon *et al.*, 1996).

1.4 EARLY EMBRYO DEVELOPMENT

During the short period from fertilisation to blastocyst formation, the embryo is transformed from a single totipotent cell to a specialised and complex organisation of cells, in preparation for implantation (Figure 1.2). In the mouse this process is complete within 4 days (Chavez, 1984).

1.4.1 Fertilisation

The beginning of new life in mammals occurs at fertilisation. Although much is known about the general requirements for fertilisation, the details remain unclear (Yanagimachi, 1988). Sperm leaving the testis do not have the ability to fertilise an egg and must first undergo maturation (capacitation) in the female reproductive tract. On reaching the fallopian tube (or oviduct), binding of sperm to the innermost extracellular membrane (zona pellucida; ZP) of the egg activates the enzymes at the sperm head (acrosome reaction). Sperm binding to the ZP, a non-covalently linked complex of sulphated glycoproteins, appears to be a carbohydrate-mediated event (Tulsiani *et al.*, 1997; Poirier & Kimber, 1997). Once a single sperm penetrates the ZP, the matrix changes to prevent adhesion or penetration of other sperm.

Evidence suggests that binding of a sperm to the egg plasma membrane is mediated by integrins (Sueoka *et al.*, 1997; Yoshimura, 1997; Bronson & Fusi, 1996). The egg cell membrane possesses a range of integrin subunits, including $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, αv , $\beta 1$, $\beta 3$ and $\beta 5$ (Tarone *et al.*, 1993; Evans *et al.*, 1995; Campbell *et al.*, 1995b). Sperm express vitronectin (VN) at their surface, and acquire fibronectin (FN) following capacitation, both of which are integrin ligands. A broad-range anti-integrin antibody has been shown to greatly reduce sperm-egg binding, as have soluble RGD (arginine-glycine-aspartic acid) peptides (Fusi *et al.*, 1992) in some species. $\alpha 6\beta 1$ is the only integrin conserved on the egg plasma membrane in all mammals examined, and a function blocking antibody to this integrin completely abolishes sperm binding in mice. One candidate mechanism for cell-cell binding using $\alpha 6\beta 1$ (or other integrins) involves family members of the cell-surface proteins which possess a disintegrin and a

metalloproteinase domain and are termed ADAMs (Wolfsberg & White, 1996; Bigler *et al.*, 1997). ADAMs contain four potential functional regions: a metalloproteinase-like proteolytic domain, an adhesion domain (containing an RGD site), a membrane fusion site, and a cytoplasmic signalling domain. Fertilin α and β (ADAMs 1 and 2) form heterodimers on the surface of sperm and mediate sperm-egg binding and fusion. Fertilin β appears to bind integrins, and is thought to be a ligand for $\alpha 6 \beta 1$. Fertilin α is proposed to mediate fusion of the sperm and egg plasma membranes (Huovila *et al.*, 1996). Sperm also express integrins and these appear to be dynamically regulated with sperm maturation. Expression of $\alpha 5$ and $\beta 1$ coincides with capacitation, while αv and $\beta 3$ are substantially increased following the acrosome reaction (Fusi *et al.*, 1996).

Following sperm-egg fusion, the egg is activated by explosive intracellular Ca^{2+} waves. This initiates Na^+/H^+ exchange, thereby elevating intracellular pH. The egg is now irreversibly activated, allowing protein and DNA synthesis with stimulation of metabolic pathways. The pronuclei of the two gametes lose their nuclear membranes and their chromosomes mingle in preparation for the first mitotic division. At this stage fertilisation has ended and embryonic development begins.

1.4.2 Pre-implantation embryo development

As the embryo descends through the fallopian tube (or oviduct) towards the uterine cavity it must undergo growth and differentiation before it becomes capable of implantation. During cleavage, the first embryonic cell undergoes several mitotic divisions producing blastomeres of equal and increasingly smaller size (Figure 1.2). These cells are totipotent and are weakly held together by ionic attraction of their plasma membranes. Early embryonic development requires a switch from maternal to embryonic transcription. This is seen from the 2-cell stage in the mouse (Johnson *et al.*, 1984), and slightly later at the 4 to 8-cell stage in humans (Braude *et al.*, 1988).

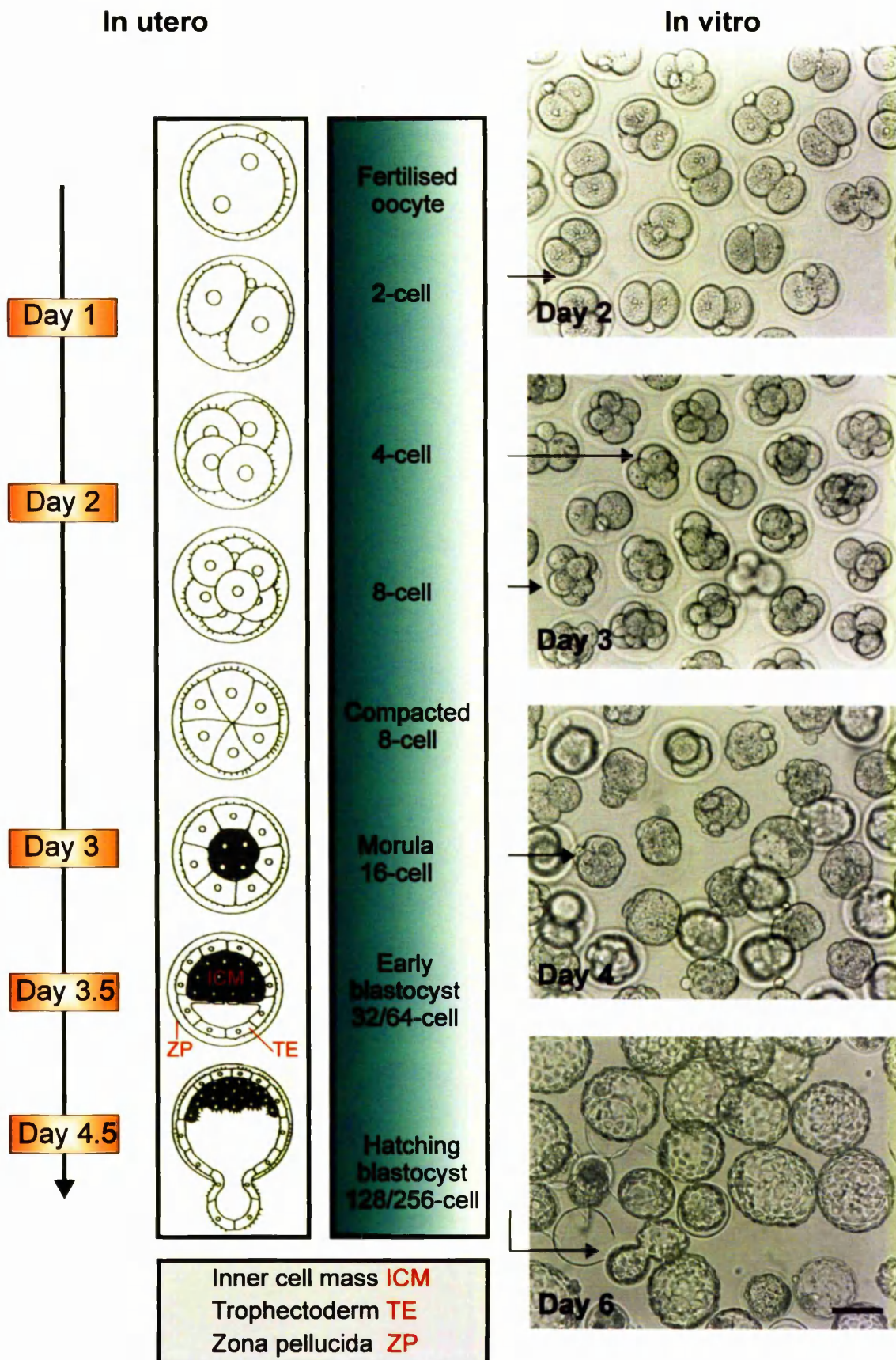


Figure 1.2 Early embryo development in the mouse
Based on Collins & Flemming, 1995; scale bar = 100µm.

Once embryos reach the 8-cell stage they undergo compaction, in which cells flatten and become tightly associated with each other (Chavez, 1984). From this stage and over the next 24 hours an array of junctional complexes develop between the lateral borders of the outer embryonic cells (Figure 1.2; Collins & Fleming, 1995; Fleming *et al.*, 1994, 1993, 1992). The first cell-cell adhesion event at compaction is mediated by E-cadherin (uvomorulin; Hyafil *et al.*, 1980). These calcium-dependent molecules mediate homophilic binding, and are linked to the actin cytoskeleton via catenins (Takeichi, 1988). Initiation of these 'adherens junctions' coincides with E-cadherin phosphorylation. Cell attachment by E-cadherin binding is synchronised with polarisation of outer embryonic cells.

This first differentiation step of embryogenesis forms the trophectoderm epithelium, the lineage which later becomes the placenta. Meanwhile the inner cell mass (ICM) which will form all embryonic tissues remains non-polar and totipotent. Adherens junctions mediate cell attachment between cells of both the trophectoderm and ICM lineages. Mouse embryos possessing a homozygous null mutation for E-cadherin undergo compaction (using residual maternal E-cadherin), but fail to form trophectoderm or a blastocyst cavity and die before implantation (Larue *et al.*, 1994).

Maximising the area of cell-cell contact during pre-implantation development appears to utilise various cell adhesion systems, including cell surface carbohydrates and lectins (Poirier & Kimber, 1997; Kimber, 1990; Kimber *et al.*, 1993). Two cell surface molecules which support cell-cell adhesion are CD44 and neuronal cell adhesion molecule (N-CAM). Both are present at the cell surface of oocytes and through pre-implantation embryo development (Campbell *et al.*, 1995a; Kimber *et al.*, 1994a). Morula stage embryos produce laminin (LM), which is located at intercellular borders. Here, LM may consolidate the increased intercellular apposition seen during compaction (Cooper & MacQueen, 1983). Null mutation of the LM C1 gene leads to loss of the LM γ 1 chain present in most forms of LM. Null mutant embryos complete pre-implantation development and do implant, but fail at around day 6.5 of development (Dr D. Edgar, personal communication). In human and mouse many integrin cell adhesion molecules are

constitutively expressed during pre-implantation development, while others are developmentally regulated (Campbell *et al.*, 1995b; Sutherland *et al.*, 1993), described further in section 1.7.6. The expression of integrin ligands, ECM molecules, in pre-implantation embryos is described in detail in section 1.8.1. Various integrin knockouts have been performed, but no single deletion has shown a phenotype in pre-implantation embryos (Fassler *et al.*, 1996; Hynes, 1996). Increased cell-cell contact allows intercellular communication, facilitated by expression of gap junction proteins (connexins; Watson, 1992).

As the embryo progresses from compaction, through the 16-cell stage (morula) to the 32-cell stage (early blastocyst; Figure 1.2), it accumulates and assembles tight junction components. This apicolateral junction is restricted to trophectoderm cells. Initial focal membrane-contact sites spread to become a lattice of linear membrane associations. Tight junctions consist of occludin branching between cells, with associated components (e.g. zona occludin 1; ZO-1 and cingulin) linked to the actin cytoskeleton. Mature tight junctions provide transepithelial resistance which allows accumulation of blastocoele fluid. To create the fluid filled blastocyst cavity, active transport of fluid (by Na^+/K^+ -ATPase activity) and its retention within the epithelial barrier is required (Watson, 1992). Closely coordinated with blastocoele formation is the appearance of desmosomes, punctate membrane contacts, at the lateral surface of the trophectoderm (Fleming *et al.*, 1991). These adhesion plaques consist of desmosomal cadherins (desmoglein and desmocollin) spanning adjacent cells, and intracellular components (desmoplakin and plakoglobin) linking the structures to the intermediate filament network.

Mammalian pre-implantation embryos can develop from a single cell to a blastocyst in simple media (Biggers *et al.*, 1993). However, embryo growth rate and cell number is lower in vitro, indicating that the maternal environment enhances development (Bowman & McLaren, 1970). Rabbit embryos in vitro develop at a rate closer to that in utero when cultures are supplemented with appropriately staged uterine flushings (Fischer *et al.*, 1990). In vitro, embryos

grown at higher density show increased survival (Paria & Dey, 1990), indicating that they secrete development-promoting factors into the medium. Growth factors, cytokines and their receptors are synthesised by the developing embryo, these are likely to be important for promoting pre-implantation development (Giudice & Saleh, 1995; Paria & Dey, 1990).

By day 4.5 of development (in the mouse) an embryo has undergone 7 or 8 rounds of cell division and consists of a layer of fully polarised trophectoderm surrounding cells of the embryo proper, some of which have undergone differentiation to become parietal endoderm (Collins & Fleming, 1995). It is at this stage that the blastocyst 'hatches' from the ZP (Figure 1.2) and is able to commence implantation into a receptive uterus.

1.5 IMPLANTATION

Once an embryo reaches the blastocyst stage (section 1.4.2) it must become associated with the receptive endometrium (section 1.3.2) for embryo development to continue. Implantation is thought to involve a precisely controlled cascade of adhesive interactions which are subsequently required for placenta formation (in human and rodents), and ultimately a successful pregnancy.

1.5.1 Mechanisms of implantation

Implantation can be divided into three stages, as follows:

Apposition

Rodent blastocysts orientate along the anti-mesometrial surface of the endometrium with their abembryonic pole closest to this surface (Mossman, 1987); human blastocysts appear to orientate the embryonic pole nearest to the endometrial surface (Knoth & Larsen, 1972; Lindenberg *et al.*, 1992). Approach of the blastocyst to the apical uterine epithelium is facilitated by collapse of the uterine lumen around the embryo (Finn, 1977) and blastocyst expansion (Borland & Tasca, 1974). Before the embryo can begin attachment it must shed its ZP. In rodents this requires enzymatic action of uterine secretions (Rosenfeld & Joshi,

1977), and in some species (including human) trophoderm projections are thought to facilitate ZP rupture (Gonzales *et al.*, 1996). Apposition of the blastocyst to the luminal epithelium of the endometrium allows interdigitation of the microvilli of the two apical surfaces, thus commencing adhesion (Nilsson, 1966; Hedlund & Nilsson, 1971)

Adhesion

In rodents it is well established that adhesion of the blastocyst to the endometrial epithelium is dependent on a surge of oestrogen (sections 1.2.2, 1.3.2). The adhesion phase of implantation is thought to involve a succession of adhesive interactions, the precise nature of which are still unclear. Both, positive and negative modulators of adhesion may be involved (Carson *et al.*, 1992a). The various families of molecules implicated in blastocyst attachment are examined in section 1.6.

Extension of the attachment site

Species differences exist in the morphology of trophoblast-endometrium interaction following embryo attachment, with respect to the existence or extent of trophoblast penetration into the uterine stroma, as determined by ultrastructural examination of early implantation sites (Schlafke & Enders, 1975). In non-invasive implantation (e.g. pig, cow), the basement membrane of the uterine epithelium is not breached; the site of embryo attachment spreads laterally along the surface of the endometrial epithelium forming an 'epithelio-chorial' placenta (Lindenberg, 1991). Invasive implantation allows placental tissue to make direct contact with the maternal blood, and is termed 'haemo-chorial' placentation. Three types of invasive implantation can be distinguished by observing the subsequent involvement of the uterine epithelium following initial blastocyst attachment (Table 1.4).

Table 1.4 Alternative mechanisms of trophoblast invasion

'Intrusive' implantation (e.g. ferret, rhesus monkey)

Trophoblast penetrates between the cells of an intact uterine luminal epithelium to the underlying basal lamina, which it then extends beneath.

'Displacement' implantation (e.g. mouse, rat)

The uterine epithelium is readily released from the underlying basal lamina, facilitating the spread of trophoblast through and beneath the epithelium.

Erosion of the basal lamina is implemented by the underlying decidual cells.

'Fusion' implantation (e.g. rabbit, ruminants)

Trophoblasts fuse with individual cells of the uterine luminal epithelium in order to penetrate it.

Fusion forms a mixed syncytium containing nuclei of maternal and fetal origin, which then exhibits an invasive phenotype and penetrates the basal lamina.

Schlafke & Enders, 1975; Schlafke *et al.*, 1985; Blankenship & Given, 1995.

Few examples of early implantation sites in human pregnancy have ever been examined, and these had progressed beyond the attachment stage of implantation (Hertig *et al.*, 1956; Knoth & Larsen, 1972). Extrapolations from implantation in closely related primate species have been made with caution (Ramsey *et al.*, 1976). Most information regarding human implantation has been gained from in vitro models using human embryos generated in in vitro fertilisation (IVF) treatment cycles (section 1.5.2; Lindenberg *et al.*, 1992). In vitro human blastocysts attach to monolayers of human uterine epithelium via their embryonic pole, and trophoblasts send cytoplasmic projections between epithelial cells causing disruption of the monolayer and stacking of the uterine epithelial cells (Lindenberg *et al.*, 1989; Lindenberg, 1991). Although this in vitro environment lacks many features of the uterine milieu, such studies strongly suggest that human blastocysts exhibit intrusive implantation (Lindenberg, 1991).

1.5.2 In vitro models for implantation

Various approaches have been used to study implantation in vitro (Kimber *et al.*, 1993; Lindenberg *et al.*, 1992). Freshly isolated pieces of uterine tissue have been collected at precise stages of hormonal differentiation, without significant disruption of tissue architecture, and blastocysts have been placed on them. Once isolated these organ cultures rapidly degenerate, and embryos tend to attach non-specifically to necrotic cells (Glenister, 1961). Enzymatic release of the endometrial epithelium from uteri provides a cell suspension which can be seeded onto plastic, cultured in simple nutrient medium, and onto which blastocysts can then be placed. Comparative studies of mouse, human and cow implantation in vitro adds weight to the value of this technique (Lindenberg *et al.*, 1989). When plated directly onto plastic the uterine epithelial monolayer does not retain a polarised phenotype and is therefore somewhat abnormal (Kimber *et al.*, 1993). Great progress has been made with the development of polarised primary cultures of uterine epithelial cells. Initially developed in the rat (Glasser *et al.*, 1988; Glasser & Mulholland, 1993), these cultures retain steroid hormone responsiveness. Similar cultures can be prepared from mouse and human endometrium (Kimber *et al.*, 1993; Schatz *et al.*, 1990). The maintenance of

epithelial polarity is achieved by seeding cells onto three-dimensional matrices of ECM components, restoring the epithelial basement membrane that exists in utero. Further advances in studying implantation in vitro are expected when uterine epithelial cells are co-cultured with uterine stromal cells, although so far such cultures have met with limited success (Kimber *et al.*, 1993).

The interaction between an embryo and the uterine stroma following initial invasion through the epithelial layer is also of considerable interest. To study this in vitro, blastocysts have been added to stromal cell cultures (Aplin & Glasser, 1994). One limitation of this model (and those using epithelial monolayers) is the difficulty of visualising the early stages of implantation and an inability to distinguish between uterine and embryonic cells (Farach *et al.*, 1987). It is of great benefit to study the molecular basis of trophoblast differentiation and function free from the constraints of the maternal environment. A reductionist and highly reproducible approach is to culture mouse blastocysts on ECM substrates. In its simplest form this involves culturing blastocysts in serum-containing medium (Sherman & Salomon, 1975), where the substrate is composed mainly of VN (Hayman *et al.*, 1985). The formulation of a biochemically defined medium capable of supporting trophoblast outgrowth in the absence of serum has allowed the role of specific substrates to be studied (Armant *et al.*, 1986a). Careful examination of the interaction between the trophoblast and molecules usually present at the apical surface of uterine epithelium, or within the epithelial basement membrane or stromal ECM are now possible. For example, these have provided evidence for the role of proteoglycan interactions and integrin-ECM binding in implantation (sections 1.8.2). This model of implantation was chosen for use in this study to further examine the expression (Chapter 4) and function (Chapter 5) of integrins in trophoblast outgrowth in vitro. Placing blastocysts on mixed substrates would allow ligand preferences and multi-step interactions to be examined, so ECM extracted from endometrium has also been used to investigate trophoblast outgrowth (section 1.8.2; Armant & Kameda, 1994).

1.5.3 Placentation

Formation of a haemo-chorial placenta, as is seen in mouse and more extremely in humans, allows fetal trophoblasts to be in direct contact with the maternal blood. This is required for exchange of oxygen, nutrients and waste products between a mother and her growing fetus (Cross *et al.*, 1994). Development of the placenta requires trophoblast cells to migrate and differentiate into various 'cytotrophoblast' (CTB) lineages (section 1.7.6; Vicovac *et al.*, 1995; Aplin, 1991; Burrows *et al.*, 1996). CTB invasion and differentiation is under very strict regulation. If CTB invasion is insufficient the embryo will not receive enough nutrients for growth, conversely if CTB invasion is unlimited it will extend beyond the endometrium akin to tumour invasion. Complex adhesive interactions occur between CTB populations and the endometrium (Aplin, 1997; Damsky *et al.*, 1993; Burrows *et al.*, 1996). These are thought to be regulated by changing repertoires of integrin expression by CTB (section 1.7.6) as they travel through an evolving extracellular environment (section 1.8.1) and then cease migration at the appropriate point. Placentation, like implantation, requires precise regulation, in part mediated by local soluble factors of fetal and maternal origin (1.5.4).

1.5.4 Local regulators of implantation

Many cytokines and growth factors with local autocrine/paracrine effects are expressed in the endometrium and embryo, some of which are critical for implantation (Tabibzadeh & Babaknia, 1995; Stewart, 1994; Giudice & Saleh, 1995; Simon *et al.*, 1996; Simon, 1996; Haimovici & Anderson, 1993).

Leukaemia inhibitory factor (LIF) is produced by the uterine epithelium, under maternal control in mouse and human (Bhatt *et al.*, 1991; Shen & Leder, 1992; Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994). LIF receptor expression is seen at the endometrial epithelium (Cullinan *et al.*, 1996) and in blastocysts (Charnock-Jones *et al.*, 1994). In mice lacking LIF gene function it has been shown that endometrial LIF expression is critical for implantation (Stewart *et al.*, 1992). Disruption of the gene encoding colony stimulating factor 1 (CSF-1) also affects fertility in mice. In these animals a decreased implantation rate is seen,

and animals born from successful pregnancies often had uterine abnormalities (Pollard *et al.*, 1991). Many other cytokines/growth factors or their receptors have been knocked out in mice without reproductive phenotype. These include isoforms of transforming growth factors α and β (TGF α ; Luetke *et al.*, 1993; TGF β ; Shull *et al.*, 1992), interleukin -6 (IL-6; Kopf *et al.*, 1994), interleukin -1 receptor (IL-1R; Abbondanzo *et al.*, 1996), insulin-like growth factor receptors (IGF-R; Baker *et al.*, 1993; Lui *et al.*, 1993), and tumour necrosis factor receptor (TNF-R; Pfeffer *et al.*, 1993).

The lack of reproductive phenotype in knockouts of many of these local factors or their receptors is quite surprising as most are present in the endometrium. A regulated expression pattern during the endometrial cycle is seen for IGF-I (Kapur *et al.*, 1992), TNF- α (Hunt *et al.*, 1992), TGF α (Tabibzadeh & Sun, 1992), platelet derived growth factor (PDGF; Grey *et al.*, 1995) and basic fibroblast growth factor (bFGF; Rider & Psychoyos, 1994). Several factors and/or their receptors are upregulated around the time of implantation, including IGF binding protein I (IGF BP-I; Giudice, 1994), IL-1 (De *et al.*, 1993; Choudhuri & Wood, 1993; Simon *et al.*, 1994; Tabibzadeh & Sun, 1992). It is likely that there is extensive cross-over between the function of these factors or their receptors.

Several epidermal growth factor (EGF) family members (including amphiregulin; AR) are implicated in reproductive development and implantation (Tsutsumi & Oka, 1987; Nelson *et al.*, 1991; Ignar-Trowbridge, 1992). AR expression is greatly elevated throughout the uterine epithelium on day 4 of pregnancy in the mouse, under the control of progesterone, and during pre-implantation development (Das *et al.*, 1995; Tsark *et al.*, 1997). It accumulates in the luminal epithelium at sites of blastocyst attachment and is then downregulated following the initial attachment reaction. AR preferentially activates EGF receptors (EGF-R) of the uterus over those of the blastocyst (Das *et al.*, 1995). In mice lacking functional EGF-R, strain-dependent effects are seen. These range from degeneration of ICM resulting in peri-implantation lethality to post-natal death

following multi-organ abnormalities (Threadgill *et al.*, 1995; Sibilia & Wagner, 1995).

Many factors have also been implicated in early embryo development and blastocyst formation, including EGF (Paria & Dey, 1990), LIF (Lavranos *et al.*, 1995), IL-1 (Simon *et al.*, 1994), PDGF and TGF β 1 (Paria *et al.*, 1992; Giudice & Saleh, 1995). Trophoblast function is also locally regulated. For example, EGF promotes trophoblast outgrowth in vitro (Das *et al.*, 1994) and increases production of proteinases (Harvey *et al.*, 1995; Zhang *et al.*, 1996). LIF modulates proteinase expression, human chorionic gonadotrophin (hCG) production and FN secretion (Harvey *et al.*, 1995; Nachtigall *et al.*, 1996). Also TNF α reduces motility of transformed trophoblasts on LM, without altering cell adhesion or β 1 integrin expression (Todt *et al.*, 1996). Clearly there are many local regulators of implantation and these modulate many functions of embryonic and maternal cells (Simon *et al.*, 1996).

1.6 BLASTOCYST ATTACHMENT

In all mammals, implantation (section 1.5) involves attachment of the trophoblast of the blastocyst to the luminal epithelium of receptive endometrium (section 1.3.2). This is irrespective of inter-species variations in the levels and ratios of oestrogen and progesterone, endometrial ultrastructure, blastocyst size, and the existence or extent of subsequent trophoblast invasion. Blastocyst attachment has been likened to the adhesive cascade of the immune system, where mobile leukocytes bind to the endothelium at sites of inflammation (Kimber *et al.*, 1994b). Here, initial carbohydrate interactions are reinforced by binding of integrins to cell surface and extracellular ligands (Brown, 1997). It seems likely that blastocyst attachment also involves a succession of adhesive events, as many molecules have been implicated in this precisely regulated phenomenon.

1.6.1 Glycoconjugates

Carbohydrate epitopes displayed by extended structures of the epithelial glycocalyx may be the first cell-surface uterine components encountered by an

implanting embryo (Carson *et al.*, 1994). Several carbohydrate epitopes (e.g. H-type-I, Lewis^y (Le^y) antigens) have been suggested to mediate embryo attachment, and these are displayed by a diverse range of molecules including mucins (e.g. MUC1) and proteoglycans (e.g. heparan sulphate proteoglycan; HSPG). Receptors for carbohydrate epitopes include other carbohydrate antigens as well as certain proteins termed lectins (Poirier & Kimber, 1997; Barondes *et al.*, 1994).

H-type-I epitope

The rodent tissue antigen H-type-I (Fuc α 1-2Gal β 1-3GlcNAc β 1-Gal) is a steroid-regulated carbohydrate structure (Kimber & Lindenberg, 1990; Kimber *et al.*, 1995) expressed at the apical surface of mouse endometrial epithelium. By day 3 of pregnancy H-type-I is strongly expressed, becoming patchy around the time of implantation, and virtually absent on day 6 (Kimber *et al.*, 1988). The activity of α (1-2)-fucosyltransferase (α (1-2)-FT), an enzyme capable of catalysing the final step in H-type-I epitope formation, has been investigated in uterine luminal epithelium. α (1-2)-FT activity closely reflects H-type-I expression (White & Kimber, 1994; Kimber & Sidhu, 1997). Lacto-N-fucopentaose (LNF-1), an oligosaccharide which carries the H-type-1 epitope, partially inhibits attachment and outgrowth of mouse embryos to cultured uterine epithelial monolayers (Lindenberg *et al.*, 1988). Furthermore, a receptor for the H-type-I epitope is expressed by mouse blastocysts, first seen following hatching from the ZP. It is restricted to the abembryonic trophectoderm (Kimber & Sidhu, 1997; Lindenberg *et al.*, 1990; Yamagata & Yamazaki, 1991).

The exact nature of the embryonic H-type-I receptor has not yet been determined, although one candidate is the Le^y carbohydrate epitope (Fuc α 1-2Gal β 1-4[Fuc α 1-3]GlcNAc; Zhu *et al.*, 1995). Immunolocalisation has shown that Le^y is expressed by the trophectoderm of the blastocyst and the uterine epithelium in mice (Zhu *et al.*, 1995). Le^y disappears from the trophectoderm following attachment, as embryos begin to outgrow in vitro (Fenderson *et al.*, 1986). In order to examine the potential role of Le^y in implantation, an anti- Le^y

monoclonal antibody was injected into the uterine lumen of mice at days 3 to 5 of pregnancy. Injection of anti-Le^y shortly before the expected time of embryo attachment (day 4 of pregnancy) was found to significantly reduce the number of implantation sites seen when compared to control uteri injected with saline (Zhu *et al.*, 1995).

Mucins

MUC1 is a cell surface associated mucin which is downregulated by progesterone in the mouse uterus around the time of implantation (Valdizan *et al.*, 1992; Braga & Gendler, 1993; Surveyor *et al.*, 1995). Mouse uterine epithelial cells in vitro downregulate MUC1 via normal metabolic processing involving cleavage of the MUC1 ectodomain (Pimental *et al.*, 1996). Removal of this mucin during the receptive period may expose previously hidden cell adhesion molecules at the uterine epithelial cell surface, making them available to an implanting embryo. Indeed, overexpression of MUC1 in transformed cells has been shown to interfere with cell adhesion by inhibiting integrin-extracellular matrix interactions (Wesseling *et al.*, 1995). Paradoxically, MUC1 is upregulated in receptive human endometrium, present both at the epithelial cell surface and in uterine secretions (Hey *et al.*, 1994, 1995). Therefore a role for MUC1 in human endometrium could involve the display of sialylated carbohydrate epitopes (Hey & Aplin, 1996) for which the embryo may have receptors, thus facilitating implantation. Clearly there may be species differences in the control of MUC1 or its role in implantation (Aplin *et al.*, 1998).

Heparan sulphate proteoglycans

A heparin-binding form of epidermal growth factor (HB-EGF) is expressed in human endometrium throughout the menstrual cycle; with levels increasing from the proliferative to secretory phase, with highest expression during the receptive phase (Birdsall *et al.*, 1996; Yoo *et al.*, 1997). HB-EGF is also expressed at the apical surface of mouse uterine luminal epithelium; mRNA appears approximately 7 hours prior to blastocyst attachment only at the site of embryo apposition, and is induced by the nidatory oestrogen surge (Das *et al.*, 1994).

HB-EGF is synthesised in a transmembrane form (HB-EGF-TM) that can be proteolytically released as a soluble factor, both of which can bind cell surfaces by interaction with EGF receptors (EGF-R; Higashiyama *et al.*, 1993) or HSPG. HB-EGF-TM is present on the uterine luminal epithelium at implantation, and can support blastocyst attachment in vitro (Raab *et al.*, 1996). This interaction appears to utilise both the HSPG and EGF-R binding sites of HB-EGF (Raab *et al.*, 1996). HSPG is expressed apically by the trophectoderm of mouse blastocysts, with the acquisition of attachment competence (Carson *et al.*, 1993) under oestrogenic control (Smith *et al.*, 1997). Blastocysts also express EGF-R at this surface (Dardik *et al.*, 1992) under the control of maternal steroid hormones (Paria *et al.*, 1993a). Interaction between HB-EGF and HSPG/EGF-R provides a potential mechanism by which blastocyst attachment is precisely regulated (Raab & Klagsbrun, 1997).

Several groups have investigated the role of embryonic HSPG in embryo attachment and outgrowth on ECM substrates in vitro (section 1.8.2). The attachment phase of this process is disrupted when embryos are treated with heparinase (Farach *et al.*, 1987; Carson *et al.* 1990a). HSPG also mediates interaction between placental (JAR) and uterine epithelial carcinoma cell lines (Rohde & Carson, 1993). A high affinity heparin/heparan sulphate interacting protein (HIP) has been identified on the surface of human uterine epithelial cells and cell lines (Wilson *et al.*, 1990; Liu *et al.*, 1996b; Rohde *et al.*, 1996). HIP binds JAR cell HSPG (Liu *et al.*, 1997) at a site disrupted by treatment with heparanase - an enzyme that degrades glycosaminoglycan chains of HSPG and is associated with invasive behaviour of cells (Marchetti *et al.*, 1997).

1.6.2 Cell adhesion molecules

Trophinin, an intrinsic membrane protein, when associated with the cytoplasmic protein tascin, produces a novel adhesion complex which may be involved in implantation (Fukuda *et al.*, 1995). These proteins have very limited tissue distribution and have been identified in human endometrium, where they are expressed only during the secretory phase by the luminal epithelium. They have

also been localised to the trophoctoderm surface of macaque blastocysts, concentrated at the embryonic pole. Both molecules are present at macaque implantation sites and are expressed by both embryonic and endometrial cells. Studies utilising tumour cell lines expressing the two proteins show that trophinin mediates homophilic binding only when associated with tasin, and supports cell-cell adhesion of teratocarcinoma (embryonic) cells to endometrial carcinoma cells (Fukuda *et al.*, 1995).

CD44 represents a large family of cell adhesion molecules that arises by alternative splicing (Mackay, 1994). These bind numerous ligands including glycans, sialylated oligosaccharides (e.g. Le^x) and ECM components (reviewed by Aplin, 1997). CD44 is expressed in pre- and peri-implantation stage human embryos (section 1.4.2; Campbell *et al.*, 1995a). The expression of CD44 in human endometrium is upregulated during the secretory phase (Albers *et al.*, 1995). Two isoforms of CD44 (CD44E, CD44H) are differentially expressed between the endometrial epithelium and stroma (Behzad *et al.*, 1994). Epithelial CD44 is potentially able to bind embryonic ECM components (section 1.8.1) or glycans (section 1.6.1).

ECM components that are not themselves membrane-bound may act as bridging molecules between the embryonic and maternal cell surface, at both blastocyst attachment and subsequent invasion (section 1.8.2). Several ECM molecules are expressed at the surface of the trophoblast or endometrial epithelium (section 1.8.1). In addition to possessing binding sites for heparin-containing molecules (e.g. HSPG) and CD44, ECM proteins are ligands for members of the integrin family of cell adhesion molecules (sections 1.7.1, 1.7.2). Integrin-ECM interactions are responsible for fundamental and diverse cellular functions, and are described in detail in section 1.7. Notably, integrins of the α_v subfamily have been implicated in blastocyst attachment, as they are apically expressed by uterine epithelial cells (Aplin *et al.*, 1996; Lessey *et al.*, 1992). Furthermore, several integrins are present at the trophoctoderm of peri-implantation stage blastocysts (Sutherland *et al.*, 1993).

1.7 INTEGRIN CELL ADHESION MOLECULES

Integrins are a large family of cell adhesion molecules involved in cell-cell and cell-ECM contact. They are non-covalently linked $\alpha\beta$ heterodimers, where each subunit consists of a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail (Figure 1.3; Hynes, 1987; Albeda & Buck, 1990). So far, 16 α and 8 β subunits forming at least 22 functional integrin dimers with diverse ligands have been identified (Zheng *et al.*, 1997). The α_v and β_1 integrin subunits both associate with numerous different subunits, producing two major integrin families with a multitude of ligands (Table 1.5). Other integrin subunits are more restricted, often having just one binding partner (Ruoslahti & Pierschbacher, 1987; Ruoslahti *et al.*, 1994). Intracellularly integrins are linked to the actin cytoskeleton and mediate second messenger signalling cascades. When dimerised, integrins at their extracellular face can interact with ligands on the surface of cells and within the extracellular environment. Therefore bi-directional communication between the inside and outside environments of cells is possible through integrins (Humphries, 1996; Dedhar & Hannigan, 1996). In comparison to the advances made in understanding the intracellular signalling pathways activated upon integrin ligation ('outside-in' signalling), considerably less is known about the regulation of ligand binding as dictated from within the cell. The interplay between these two mechanisms governs the function of integrins and their role in fundamental processes such as cell division, differentiation and migration.

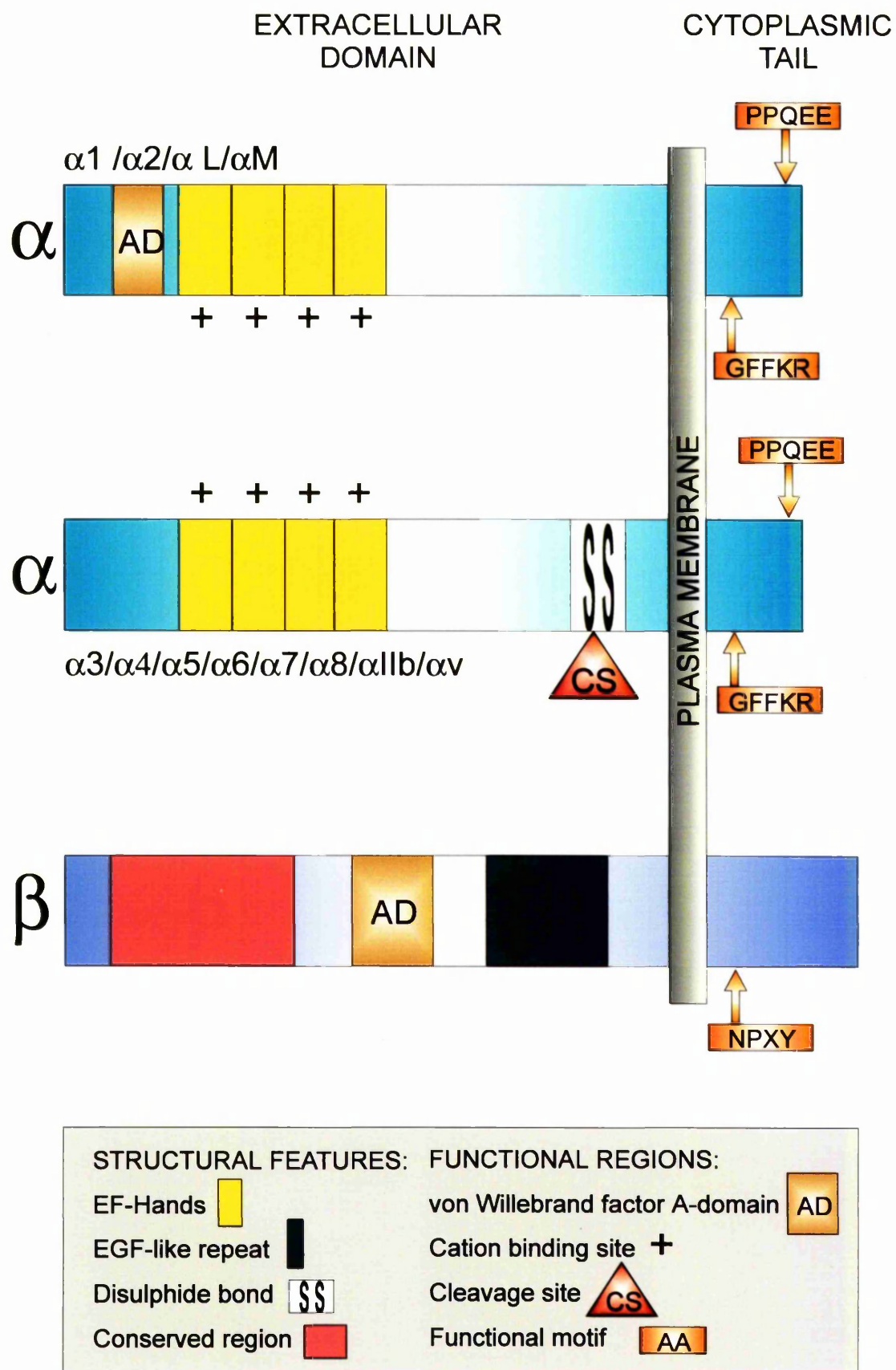


Figure 1.3 A summary of integrin subunit structure

Adapted from Tuckwell *et al.*, 1993.

1.7.1 Integrin-ligand diversity

Integrins bind a diverse array of ligands including ECM proteins (Table 1.5, Figure 1.4 in section 1.8), plasma proteins and integral membrane proteins (Haaz & Plow, 1994). Many integrins bind more than one of these ligands, and some of the ligands bind several integrins, using either the same or distinct recognition sequences. Furthermore, binding of integrins to one type of ligand can regulate the function of other integrins (Wright *et al.*, 1993; Pjuan-Thompson & Gladson, 1997; Simon *et al.*, 1997b).

ECM integrin ligands - a central role for amino acid recognition sequences

Integrins generally recognise short amino acid sequences in their ligands, which include a key acidic residue that is essential for ligand binding. Diverse integrin ligands (e.g. FN, echistatin, viruses) often share the common RGD amino acid sequence, which is presented in a topographically similar manner. Other integrin ligands (e.g. vascular cell adhesion molecule-1 (VCAM-1), E-Cadherin) share this same structural homology but not the same primary amino acid sequence (Sugimori *et al.*, 1997). Many integrin ligands contain RGD although it is not always accessible for binding. $\alpha v \beta 3$ ligands are primarily RGD containing peptides, however novel binding sequences (SAGT and NGR) have been implicated in $\alpha v \beta 3$ binding to VN and FN respectively. These sequences may act together with RGD to promote ligand binding (Healy *et al.*, 1995). The role for synergistic sites (other than RGD) have been shown in FN-mediated cell adhesion (Mould *et al.*, 1997). Several integrins ($\alpha 5 \beta 1$, $\alpha 11 b \beta 3$, $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 6$) all recognise RGD in the tenth type III repeat of FN (see Figure 1.4) but have distinct requirements for synergistic adhesion sites (Chen *et al.*, 1996). Thus, synergistic adhesion sites allow distinction between the adhesion of different integrins to the same ligand.

Table 1.5 Extracellular matrix ligands of $\beta 1$ - and αv -family integrins

| | | | | | | | | | | | |
|-------------------------------------|-------------------------------------|-----|----|----|----|----|-----|----|-----|-----|--|
| $\alpha 1\beta 1$ | | COL | LM | | | | | | | | |
| $\alpha 2\beta 1$ | $\alpha 3\beta 1$ | COL | LM | | | | | | | | |
| $\alpha 4\beta 1$ | <u>$\alpha 5\beta 1$</u> | | | FN | | | | | | | |
| $\alpha 6\beta 1$ | $\alpha 7\beta 1$ | | LM | | | | | | | | |
| $\alpha 8\beta 1$ | | | | FN | | VN | | | TN | | |
| $\alpha 9\beta 1$ | | | | | | | | | TN | | |
| <u>$\alpha v\beta 1$</u> | | COL | | FN | FB | VN | vWF | | | | |
| <u>$\alpha v\beta 3$</u> | | | LM | FN | FB | VN | vWF | TN | OST | TSP | |
| <u>$\alpha v\beta 5$</u> | | | | FN | FB | VN | | | | | |
| <u>$\alpha v\beta 6$</u> | | | | FN | | | | | TN | | |
| $\alpha v\beta 8$ | | | | | | VN | | | | | |

Collagen, COL; laminin, LM; fibronectin, FN; fibrinogen, FB; vitronectin, VN; von Willebrand factor, vWF; tenascin, TN; osteopontin, OST; thrombospondin, TSP.

Each ligand is reported to contain an arginine-glycine-aspartic acid (RGD) amino acid sequence. Integrins known to recognise RGD are underlined.

Based on: Sugimori *et al.*, 1997; Haas & Plow, 1994; Craig *et al.*, 1995; Liaw *et al.*, 1995; Humphries, 1990.

In all cases, it seems that these integrin-recognition sequences (Figure 1.4 in section 1.8) are within an exposed and conformationally mobile loop, which is accessible for ligand binding. Furthermore, it is the regions surrounding these sequences that confer ligand-receptor specificity (Sugimori *et al.*, 1997). For example, in cells possessing distinct receptors for FN and VN, a range of RGD-containing peptides had widely different capacities to interfere with integrin-ligand binding, reflecting the different affinities of the peptides for FN and VN receptors (Pierschbacher & Ruoslahti, 1987).

Cell-cell adhesion mediated by integrins

Several integrins can heterotypically bind cell adhesion molecules of the immunoglobulin superfamily (IgSF), thus mediating cell-cell adhesion. VCAM-1 binds $\alpha 4\beta 1$ or $\alpha 4\beta 7$ with equal avidity (Hedman & Lundgren, 1996). Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) however binds strongly to $\alpha 4\beta 7$, with little affinity for $\alpha 4\beta 1$. This differential binding preference is determined by regulatory residues within MAdCAM-1, adjacent to the integrin-contact sites (Newham *et al.*, 1997). A third IgSF member, neuronal L1-CAM, binds to $\alpha v\beta 3$; this is mediated by an RGD sequence on L1-CAM and is cation dependent (Montgomery *et al.*, 1996). L1-CAM also binds $\alpha 5\beta 1$, and can be shed from the cell surface and subsequently detected within the ECM (Kadmon & Altevogt, 1997). $\alpha v\beta 3$ also binds platelet endothelial cell adhesion molecule-1 (PECAM-1), in a cation-dependent manner. This interaction can be blocked by soluble RGD peptides, although PECAM-1 does not contain an RGD sequence (Piali *et al.*, 1995; Buckley *et al.*, 1996).

Additional potential integrin ligands exist within the disintegrin family of proteins, first identified in snake venom and shown to be potent inhibitors of platelet aggregation mediated by $\alpha IIb\beta 3$. Disintegrins may act competitively as integrin ligands, either in soluble form or when membrane-anchored (Weskamp & Blobel, 1994). A novel cell surface disintegrin, metargidin, possess an RGD sequence within its putative integrin-recognition domain, and also contains a proteinase

domain and a cytoplasmic tail. Disintegrins may prove to be important regulators of adhesive function (Kratzschmar *et al.*, 1995).

1.7.2 Integrin-ligand binding

Requirements for ligand binding

Transduction of information by integrins requires ligand binding, divalent cation binding and conformational change. Although the exact sites for each of these are not yet clear, functional regions within α and β subunit extracellular domains are gradually being mapped. All integrin-ligand interactions are dependent upon divalent cations, and each of the ligand-binding regions contains potential cation binding sites (Humphries, 1996). In general, ligand binding is promoted by Mn^{2+} and inhibited by Ca^{2+} . Of the various sites identified with cation binding activity it is clear that they do not have identical cation preference (Mould *et al.*, 1995a; Mould, 1996), that cation binding at one site can affect the affinity of another, and that several sites can influence ligand binding (Suehiro *et al.*, 1996). For instance, activation of $\alpha v \beta 3$ prevents occupation of a Ca^{2+} site (Pelletier *et al.*, 1996). Therefore cation-binding sites can have direct and indirect effects on ligand binding. Site-directed mutagenesis of residues involved in cation co-ordination disrupts ligand binding, as do mutations in non-cation coordinating residues (Mould, 1996).

Three major integrin-ligand binding regions have been identified by epitope mapping, chemical crosslinking of ligand peptides, and analysis of engineered and naturally occurring integrin mutants (Figure 1.3; see Humphries, 1996; Loftus *et al.*, 1994 for reviews). The first ligand-binding region is conserved between all β subunits and located within the A-domain. Recently, a distinct cation-dependent adhesion site has been described in $\beta 3$ and $\beta 5$ (Lin *et al.*, 1997). The second major ligand-binding region is conserved between all α subunits and located near to the EF-hand-like repeats. The third region, present in only a subset of α subunits, is within the A-domain (Figure 1.3; Lee *et al.*, 1995) and appears to have a dominant role. As inhibitory antibodies acting on the β subunit

have equal effect on α subunits with or without an A-domain, this region probably supplies an additional site for ligand binding.

Conformation-sensitive antibodies have recognised additional 'ligand-induced binding sites' (LIBS; Honda *et al.*, 1995; Mould *et al.*, 1995b; Bazzoni *et al.*, 1995; Du *et al.*, 1993), for example at the extreme amino terminus of $\beta 3$. As their name suggests, these LIBS appear following the conformational changes associated with integrin occupancy, such as when $\alpha v \beta 3$ binds disintegrins (Juliano *et al.*, 1996). An RGD-binding site has been localised to within the first 200 residues of the $\beta 3$ subunit. This is disrupted by point-mutations of residues which co-ordinate divalent cations (Bajt & Loftus, 1994; Smith & Cheresch, 1988; D'Souza *et al.*, 1988). Alternatively, the necessity for association between the 55kDa and 85kDa amino-terminal fragments of αIIb and $\beta 3$ respectively, has been described as a requirement for RGD binding (Lam, 1992). Differential ligand preference of αv and αIIb is determined by the amino-terminal 334 residues of the two subunits (Loftus *et al.*, 1996). In a model of integrin-ligand binding proposed by Humphries (1996), several key features are central. Integrin conformational changes occur at key 'hinge' regions, these are sensitive to minute changes in cation binding and involve both α and β subunits. Ultimately ligand binding induces a cascade of conformational changes which are propagated through the cell membrane to trigger intracellular signalling pathways.

Structural features of integrin extracellular domains

The structural features of integrin extracellular domains underlie their ligand binding capacity (Figure 1.3). At the amino-terminus of the α subunit are conserved EF-hand-like cation-binding repeats. Two other extracellular domain features appear to be mutually exclusive between subsets of integrin α subunits. A protease cleavage site, found towards the transmembrane region, allows post-translational cleavage of some α subunits (including $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, αv) by furin, to produce a disulphide bonded 2-part α subunit (Lehmann *et al.*, 1996). Subunits lacking this cleavage site (including $\alpha 1$, $\alpha 2$, αL , αM) possess a domain

with structural homology to the von Willebrand factor (vWF) A-domain (Tuckwell *et al.*, 1993). The amino-terminus of integrin β subunits contains a highly conserved motif. The membrane-proximal region of the β subunit is composed of four EGF-like repeats. Separating these two sites is a putative vWF A-domain (Humphries, 1996). Formation of an integrin dimer is cation dependent and appears to rely on interaction of the two extracellular domains (Tuckwell *et al.*, 1993). The structure of integrin cytoplasmic domains is described in section 1.7.3.

Life cycle of integrins at the cell surface

Integrins are continually synthesised, processed, transported to the cell surface, and then internalised and degraded. Rates of passage through this ongoing cycle are influenced by integrin-occupation. In non-adherent cells, surface expression of unoccupied integrins is not stabilised and integrin degradation dominates (Dalton *et al.*, 1995). When cells are adherent, it is the type of ligand available that regulates integrin expression and ultimately cell function (Delcommenne & Streuli, 1995). Human skin fibroblasts use $\beta 5$ integrins alone for VN internalisation, and the two proteins are co-localised in endocytotic vesicles (Memmo & McKeown-Longo, 1998). Astrocytoma cells on a range of substrates can internalise and degrade soluble VN via $\alpha v\beta 3$ and $\alpha v\beta 5$ (Pijuan-Thompson & Gladson, 1997). When cells are attached to FN via $\alpha 5\beta 1$, both $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute equally to VN internalisation. However, the same cells on COL or LM substrates, attached via $\alpha 3\beta 1$, use $\alpha v\beta 5$ alone for VN internalisation, although expression of $\alpha v\beta 3$ is unchanged. It seems that ligation of $\alpha 5\beta 1$ is required for $\alpha v\beta 3$ to mediate VN internalisation (Pijuan-Thompson & Gladson, 1997). Conversely, ligation of $\alpha v\beta 3$ has been shown to suppress $\alpha 5\beta 1$ -mediated internalisation of FN, without affecting $\alpha 5\beta 1$ -mediated adhesion to a FN substrate in the same cells (Blystone *et al.*, 1994). Also $\alpha v\beta 3$ regulates $\alpha 5\beta 1$ -mediated cell migration toward FN in human embryonic kidney cells (Simon *et al.*, 1997b). Thus, additional regulation of integrin function is provided by other integrins.

1.7.3 The intracellular face of integrins

Intracellular regulation of cell adhesion via integrins can occur both by controlling the repertoire of receptors available at the cell surface and by modulating the ligand affinity of integrins on display (termed 'inside-out' signalling; Williams *et al.*, 1994). Inside-out signalling involves propagation of conformational change from the cytoplasmic to the extracellular domain of integrins. This potentiation of ligand binding is induced by intracellular signalling events and ultimately regulates cell function (O'Toole *et al.*, 1994). For example, activation of integrins via their cytoplasmic tails directly stimulates FN matrix assembly (Wu *et al.*, 1996).

α subunit cytoplasmic domains

A high level of inter-species sequence homology exists between integrin cytoplasmic domains. The structure and conformation of these regions are central to the 'inside-out' transfer of information (Dedhar & Hannigan, 1996). Platelets have provided a useful tool for investigating cellular signalling, as they lack a nucleus and therefore changes cannot be attributed to transcriptional modulation. 'Inside-out' signalling is demonstrated with platelet integrin α IIb β 3 which shows low affinity for soluble fibrinogen. Following platelet activation via intracellular messages, α IIb β 3 has a greatly enhanced affinity for its ligand, which ultimately results in platelet aggregation (Bennett & Vilaire, 1979; Marguerie *et al.*, 1979). Mutation of the GFFKR sequence (Figure 1.3) of the α IIb subunit is sufficient to induce a constitutively active state, independent of the β 3 subunit (Williams *et al.*, 1994). This sequence is conserved between all α subunit cytoplasmic domains (~30 amino acids in total) and can bind calreticulin. Calreticulin may disrupt a potential salt-bridge formed between α and β subunits (Hughes *et al.*, 1996) and thereby alter their activation state (Dedhar & Hannigan, 1996). An α v subunit lacking 5 amino acids (PPQEE; Figure 1.3) is no longer able to bind FN or VN when associated with β 3. This structural motif is highly conserved and may provide a mechanism for regulating integrin function (Filardo & Cheresh, 1994). Ligation of osteopontin (OST) with α v β 3 activates a Src dependent signalling cascade (Ras pathway) by direct interaction of α v

cytoplasmic domain with *Src* (Chellaiah *et al.*, 1996). Furthermore the α_v cytoplasmic tail is required for tyrosine phosphorylation of the β_3 subunit upon ligand binding, and activation of the Ras signalling pathway (Blystone *et al.*, 1996). In general, α subunit cytoplasmic tails confer cell-type-specific integrin affinity states but can also play a direct role in signalling (Dedhar & Hannigan, 1996).

β subunit cytoplasmic domains

Integrin β subunit cytoplasmic domains (~50 amino acids) are known to interact with several intracellular proteins involved in cytoskeletal organisation and signal transduction. Most possess a conserved NPXY sequence (Figure 1.6.1) which is required for post-ligand binding events (Filardo *et al.*, 1995), including phosphorylation of focal adhesion kinase (FAK; Tahiliani *et al.*, 1997). The cytoplasmic domain alone of β_1 , β_3 or β_5 integrins is sufficient to trigger tyrosine phosphorylation of FAK (Akiyama *et al.*, 1994). A sequence of 10 amino acids from the β_1 cytoplasmic domain directly binds α -actinin and an alternative region binds talin. Both of these proteins can bind actin, either directly or via vinculin. β subunit cytoplasmic domains also bind integrin-linked kinase (ILK) and β_3 -endonexin, and are required to recruit FAK to focal adhesions (section 1.7.4). Various transmembrane adapter proteins link specific integrins to distinct intracellular pathways (Giancotti, 1997). Integrin-associated protein (IAP) is one such adapter, which co-precipitates from neutrophils with $\alpha_v\beta_3$ and regulates integrin-ligand binding (Dedhar & Hannigan, 1996; Lindberg *et al.*, 1996). Integrin chimeras have demonstrated that β_1 integrins localise to focal adhesions but β_5 integrins do not, resulting in different functions, as dictated by the cytoplasmic domain alone (Pasqualini & Helmer, 1994). β_6 has a C-terminal 11 amino acid sequence which is required to stimulate cell proliferation, but is not required for other functions of the cytoplasmic domain (Agrez *et al.* 1994).

Both α and β subunits also have potential sites of phosphorylation, although activity of these has yet to be demonstrated (Williams *et al.*, 1994). An exception is β_4 which has a cytoplasmic tail of ~1000 amino acids; which as the $\alpha_6\beta_4$

heterodimer becomes phosphorylated upon ligand binding. The $\beta 8$ subunit also has a cytoplasmic domain distinct from that of other β subunits (Nishimura *et al.*, 1994).

A further complexity is that splice variants (e.g. $\alpha 6A$ and $\alpha 6B$) of some integrin subunits ($\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 1$, $\beta 3$ and $\beta 4$) produce alternate cytoplasmic domains. These have distinct expression patterns and functions (Zheng *et al.*, 1997). For example, $\beta 1A$ and $\beta 1D$ both localise to focal adhesions (section 1.7.4). $\beta 1B$ however is not found in focal adhesions and inhibits cell adhesion and motility (Balzac *et al.*, 1994). $\beta 1C$ has growth suppressing activity (Zheng *et al.*, 1997). 'Inside-out' regulation of integrins exists, where cytoplasmic tail changes are transduced through the integrin to its extracellular domain. This adds a further dimension to the regulation of integrin ligand binding and the events that follow (Liu *et al.*, 1996a; Kieffer *et al.*, 1996).

1.7.4 'Outside-in' transduction of information through integrins

A requirement for integrin clustering

Upon ligand binding and clustering of integrins, an array of metabolic changes occurs within a cell (Table 1.6; adapted from Lafrenie & Yamada, 1996). Integrin occupancy is sufficient to produce receptor re-distribution, but integrin clustering is required for recruitment of cytoskeletal proteins and stimulation of signalling pathways (Miyamoto *et al.*, 1995). This requirement for integrin clustering has been demonstrated for integrins interacting with monovalent ligands (such as antibody Fab' fragments), which require subsequent crosslinking with secondary antibodies to initiate downstream events (Kornberg *et al.*, 1991). Many cells in vitro adhere to their ECM substrate by discrete and very close points of contact. These were first identified when the ventral surfaces of cells were examined by interference reflection microscopy and termed 'focal adhesions' (Izzard & Lochner, 1976). These are now known to result from assembly of ECM components and intracellular signalling molecules (e.g. vinculin, talin, FAK) via interaction with integrin clusters, forming a complex, highly organised and dynamic structure (Yamada & Geiger, 1997; Richardson & Parsons, 1996).

Table 1.6 Steps involved in integrin-dependent signal transduction

1. Ligand binding
2. Receptor clustering
3. Intracellular events:
 - i. Focal adhesion assembly & cytoskeletal organisation
 - ii. Activation of serine/threonine (PKC, ILK, MAPK) & tyrosine kinases (FAK)
Phosphorylation of:
 - a. Focal adhesion proteins (e.g. α -actinin, FAK, paxillin, talin, vinculin)
 - b. Src-family kinases (e.g. *Src*, *Csk*, *Fyn*, *Nck*, *Syk*)
 - c. ECM receptors (integrins)
 - iii. Increased intracellular Ca^{2+}
 - iv. Increased cytoplasmic pH
 - v. Altered inositol metabolism
4. Activation of transcription factors
5. Transcription of specific genes
6. Altered cell function

PKC, protein kinase C; ILK, integrin linked kinase; MAPK, mitogen activated protein kinase; FAK, focal adhesion kinase.

Two highly homologous integrins ($\alpha v\beta 3$, $\alpha v\beta 5$) show distinct patterns of clustering in cells adherent to VN, under the influence of Ca^{2+} , Mg^{2+} and Mn^{2+} . $\alpha v\beta 3$ forms focal adhesions (co-distributed with vinculin and talin) in the presence of Mg^{2+} or Mn^{2+} , but not Ca^{2+} . $\alpha v\beta 5$ forms focal adhesions in the presence of Mg^{2+} or Ca^{2+} ; with Mn^{2+} it clusters on the ventral cell surface (without vinculin and talin). The organisation of integrin clusters is competitively and dynamically regulated by extracellular divalent cation availability, and determines the ability to initiate signalling pathways (Stuiver *et al.*, 1996).

Intracellular signalling pathways

With focal adhesion assembly, downstream events are produced through two interconnected mechanisms involving chemical signals and structural cytoskeletal change. These can induce common signals and those that are specific for individual integrins (Giancotti, 1997; Ruoslahti, 1997; Dedhar & Hannigan, 1996; Lafrenie & Yamada, 1996; Schaller & Parsons, 1994).

Soluble ('chemical') VN interaction induces G-protein dependent pathways, independent of tyrosine phosphorylation of paxillin. In contrast, substratum-bound ('physical') VN interaction stimulates tyrosine phosphorylation of paxillin. Despite this difference, in both cases the signals are mediated by $\alpha v\beta 3$ and dependent on RGD (Aznavorian *et al.*, 1996). In endothelial cells, clustering of $\alpha v\beta 3$ when ligated to soluble multimeric (and not monomeric) VN induces tyrosine phosphorylation of several proteins (including paxillin and FAK) and is dependent on $\alpha v\beta 3$, VN and RGD (Bhattacharya *et al.*, 1995). Integrin $\alpha v\beta 5$ localises to focal adhesions in macrophages stimulated with macrophage colony stimulating factor (M-CSF) and is able to mediate adhesion to a VN substrate (De Nichilo & Burns, 1993). This induces localisation of paxillin to focal adhesions (independent of FAK) and serine phosphorylation of paxillin via protein kinase C (PKC; De Nichilo & Yamada, 1996). In some carcinoma cell lines, cell adhesion to VN is mediated by both $\alpha v\beta 3$ and $\alpha v\beta 5$. However, $\alpha v\beta 3$ is found localised at focal adhesions and linked via vinculin and talin to the actin cytoskeleton, and $\alpha v\beta 5$ is evenly distributed on the cell surface and not associated with focal

adhesion components (Wayner *et al.*, 1991). Changes in intracellular Ca^{2+} regulate many cell processes, including integrin activation (Sjaastad & Nelson, 1997). Ligation of α_v -family integrins to RGD can elevate intracellular calcium through two distinct mechanisms - by releasing it from intracellular stores and by stimulating influx through ion channels (Sjaastad *et al.*, 1996). Chemical signalling pathways are linked to the cytoskeleton and can induce structural change.

Loss of phosphatidylinositol 3-kinase (PIP-3K) activity disrupts $\alpha_v\beta_3$ -mediated adhesion of melanoma cells to VN, and causes breakdown of actin stress fibres. As integrin expression remains stable, this is probably the result of altered integrin affinity (Metzner *et al.*, 1996). Complex changes in cytoskeletal structure are initiated with cell spreading. Actin polymerises, even before any change in cell shape is seen. With initial rapid spreading, actin microfilament reorganisation occurs and then stabilises. As cell spreading slows, an increase in microtubule mass is seen, where the integrity of this component is essential in stabilising cell form (Mooney *et al.*, 1995). Specific structural changes and intracellular signalling pathways are activated on integrin-ligand binding, and these are dependent on ligand conformation, cell type, integrin type and integrin activation state.

1.7.5 Integrin-dependent signals converge with other cellular systems

Intracellular signals induced upon integrin ligation have diverse effects, including those on other cell-matrix interactions mediated by integrins (section 1.7.2). Altered cell-cell adhesion also occurs, following integrin binding to FN. This coincides with increased tyrosine phosphorylation, and downregulation of N-cadherin and β catenin at cell-cell junctions (Levenberg *et al.*, 1998). Further effects of integrin ligation are possible through the association of intracellular signalling pathways with those of growth modulators and proteinases.

Growth modulators

Integrin-induced signalling mechanisms are integrated with those of growth factors, resulting in synergistic action of the two systems (Schwartz, 1997). The effects of growth factors can modulate, and be modulated by, integrin-ligand interaction (Lafrenie & Yamada, 1996). The migratory activity of melanoma cells on VN can be mediated by $\alpha v\beta 3$ alone. For $\alpha v\beta 5$ alone to support this process, the same cells must be exposed to IGF. $\beta 3/\beta 5$ subunit chimeras demonstrated that the requirement for IGF was dictated specifically by the β subunit extracellular domain (Filardo *et al.*, 1996) and resulted in redistribution of α -actinin to membrane adhesion sites containing $\alpha v\beta 5$ (Brooks *et al.*, 1997). Platelet derived growth factor (PDGF) stimulates endothelial cell migration on VN in an RGD-dependent manner that requires $\alpha v\beta 3$ (Woodard *et al.*, 1998). Smooth muscle cells on VN are also stimulated to migrate on addition of IGF, by reduced $\alpha 5\beta 1$ and increased $\alpha v\beta 3$ activity at the cell surface (Jones *et al.*, 1996). Similarly, breast carcinoma cells migrate specifically in response to IGF on VN (via $\alpha v\beta 5$) or COL IV (via $\alpha 2\beta 1$) but not on FN or LM or via other integrins (Doerr & Jones, 1996). $\alpha v\beta 6$ is also specifically upregulated following IGF stimulation, in airway epithelium in vitro. However, both $\alpha 5\beta 1$ and $\alpha v\beta 6$ are increased on addition of EGF (Wang *et al.*, 1996).

Cytokines also modulate integrin expression. During osteoclast differentiation in vitro, IL-4 stimulates increased $\alpha v\beta 3$ at the cell surface via accelerated transcription of the $\beta 3$ gene, without altering αv transcription (Kitazawa *et al.*, 1995). In several carcinoma cell lines, stimulation with LIF or $TNF\alpha$ leads to increased attachment to FN mediated by increased surface expression of $\alpha v\beta 1$ (Heymann *et al.*, 1996). Related cytokines (granulocyte-colony stimulating factor (G-CSF) or M-CSF) can induce differential integrin expression ($\alpha v\beta 3$ or $\alpha v\beta 5$) in macrophages, resulting in alternative substrate-requirement and cell function (De Nichilo & Burns, 1993). So, the effect of growth regulators on cell adhesion and migration is mediated by expression of specific integrins, where the outcome is dependent on ligand availability. When cells are extracted with mild detergent, membrane-cytoskeleton complexes (focal adhesions; section 1.7.4) remain

intact. Isolated endothelial cell focal adhesions contain many signalling pathway components, including FAK, PIP-3K, phospholipase C and ion channels, and in addition high affinity fibroblast growth factor (FGF) receptors (Plopper *et al.*, 1995). Therefore the components of these complex signalling cascades remain structurally associated at focal adhesions, stabilised by the cytoskeleton.

Proteinases

The proteinase receptor urokinase-type plasminogen activator receptor (uPAR) is found at focal adhesions. uPAR forms a stable complex with integrins at the extracellular face, and can modulate their function. This interaction promotes adhesion to, and migration towards, VN (via uPAR-VN binding), and suppresses normal adhesive function of activated $\beta 1$ integrins (Wei *et al.*, 1996). Plasminogen activator inhibitor (PAI-1), independent of its role as a proteinase inhibitor, inhibits cell migration on VN by competing with VN for integrin-binding sites (Kjoller *et al.*, 1997). Proteolytically-active matrix metalloprotease (MMP-2) can directly bind $\alpha v \beta 3$ on the surface of invasive cells, facilitating COL adhesion and degradation. This association is independent of the MMP-2 catalytic domain and requires divalent cations (Brooks *et al.*, 1996). Clearly the intimate association of integrins, their ligands, other cell adhesion mechanisms, pericellular proteinases and local growth modulators must hold the key to the precise orchestration of cell adhesion, motility and differentiation in the rich cellular microenvironment *in vivo*.

1.7.6 Integrins in implantation

Expression and regulation of endometrial integrins

Investigation of integrin expression in normal cycling human endometrium has identified receptors that are constitutively expressed with changing steroid hormones (section 1.2.1). Some integrins however are upregulated around the time of implantation (1.3.2). At this time integrins at the apical surface of luminal epithelial cells may mediate embryo attachment (section 1.6) and stromal integrins could facilitate trophoblast invasion (section 1.5.1).

The expression of several $\beta 1$ family integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$) in human endometrium has been examined (Tabibzadeh, 1992; Bischof *et al.*, 1993; Klentzeris *et al.*, 1993; Lessey *et al.*, 1992, 1994a, 1996). All were expressed by epithelial cells. Stromal cells were positive for $\alpha 1\beta 1$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$, and each of these integrins were most strongly expressed in the basalis. Of all integrins studied, temporal changes in expression were only seen for $\alpha 1\beta 1$ and $\alpha 4\beta 1$, where $\alpha 4\beta 1$ was highest in glandular epithelium from the mid-proliferative to mid-secretory phase. Of most interest is the observation that epithelial expression of $\alpha 1\beta 1$ is restricted to the early- to mid-secretory phase and is seen in stroma only during the pre-decidual period, and therefore may play a role in implantation. Proliferative phase stromal cells in vitro are induced to express $\alpha 1\beta 1$ on supplementation with EGF, TGF α or TGF β (Grosskinsky *et al.*, 1996). Expression of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ shows no correlation with the endometrial expression of steroid hormone receptors (van der Linden *et al.*, 1995); $\alpha 1$ was not included in this investigation. Also, endometrial epithelium and stroma in vitro show no hormonal regulation of integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 4\beta 1$ or $\alpha 5\beta 1$ (Sillem *et al.*, 1997). During the first trimester of pregnancy, $\alpha 4$ is lost from glands and stroma, while $\alpha 3$ and $\alpha 6$ appear in the stroma (Ruck *et al.*, 1994; Shiokawa *et al.*, 1996b). In term decidual cells $\alpha 3\beta 1$ and $\alpha 1\beta 1$ predominate (Malak & Bell, 1994). Attachment of human CTBs to (Burrows *et al.*, 1995), and outgrowth of mouse embryos on (Shiokawa *et al.*, 1996a), human decidual cell monolayers in vitro is mediated by $\beta 1$ family integrins.

The α_v integrin subunit is present in cycling endometrium (Aplin *et al.*, 1996) with increased expression during the secretory phase. Its major binding partner β_3 is restricted to the functionalis endometrium and appears abruptly on the glandular epithelium on day 20 of the cycle, as the window of receptivity opens (Lessey *et al.*, 1992). Conflicting evidence regarding the expression of β_3 at the luminal epithelium exists (Lessey *et al.* 1992, 1994a, 1996). α_v and β_3 have also been described in proliferative and pre-decidual stroma, and first trimester glands (Lessey *et al.*, 1994a). In primary cultures of endometrial epithelium, expression of β_3 is independent of steroid regulation (Sillem *et al.*, 1997), but is upregulated by embryonic IL-1 (Simon *et al.*, 1997a). β_5 , another partner for α_v , is clearly localised to the apical surface of both glandular and luminal endometrial epithelial cells, and to the stroma. The expression of this subunit shows no significant alteration with changing steroid hormones (Aplin *et al.*, 1996). Another recent study failed to detect $\alpha_v\beta_5$ in endometrial epithelium, but described sporadic expression of $\alpha_v\beta_6$ in the luminal epithelium, with a marked reduction around the time of implantation (Lessey *et al.*, 1996). This is in contrast to previous work that has demonstrated elevated β_6 in the epithelium of secretory endometrium, highest in the functionalis layer (Breuss *et al.*, 1995). Levels of endometrial integrin mRNA through the menstrual cycle have been quantitated by RT-PCR. Here it was shown that all subunits studied (α_2 , α_3 , α_4 , α_5 , α_6 , α_v , β_1 , β_2 , β_3 , β_5) were upregulated during the secretory phase, suggesting differential control of integrin transcription and translation (Dou *et al.*, 1997). The endometrial carcinoma cell line AN3 CA is a highly migratory clone with reduced adhesion to several $\alpha_v\beta_3$ integrin ligands, when compared to normal endometrial epithelium. These cells express $\alpha_v\beta_3$ protein but it is not present at the cell surface (Yelian *et al.*, 1997). In an alternative cell line (Ishikawa), which possess functional steroid receptors, $\alpha_v\beta_3$ expression is downregulated in response to oestrogen and upregulated by EGF or TGF α (Somkuti *et al.*, 1997).

In order to further elucidate the roles of integrins (especially $\alpha_1\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$) in implantation, endometrial tissue from women of compromised fertility has been studied. In normal menopausal endometrium, expression of α_1 , α_4 and

$\beta 3$ is absent (Lessey *et al.*, 1995a). In unexplained cases of subfertility exhibiting morphologically delayed endometrial differentiation, epithelial expression of $\beta 3$ is delayed or absent (Lessey *et al.*, 1992, 1995b; Lovely *et al.*, 1997), suggesting a possible role for $\beta 3$ as a marker of endometrial secretory differentiation and potentially receptivity. Absence of $\alpha 4\beta 1$ from glandular and luminal epithelium has also been reported in unexplained infertility (Klentzeris *et al.*, 1993). Following administration of postcoital contraceptives late in the receptivity phase, endometrial expression of $\alpha 1$ and $\alpha v\beta 3$ is unchanged (Taskin *et al.*, 1994). Women exposed to diethylstilbestrol (a potent non-steroidal oestrogen) in utero have a higher incidence of subfertility in adulthood. Endometrial samples taken from these women show no notable abnormalities in integrin expression, with the exception of slightly higher levels of $\alpha 5$ and αv in the stroma (Castelbaum *et al.*, 1995). Various groups have investigated whether changing patterns of integrin expression could be a causative factor or a marker of endometriosis, a condition associated with reduced fertility in which endometrial fragments form ectopic adhesions. A significant reduction or absence of $\beta 3$ subunit expression in eutopic endometrium has been correlated with advancing stage of disease (Lessey *et al.*, 1994b; Rai *et al.*, 1996). Also, increased $\alpha 3$ and decreased $\alpha 6$ has been reported (Rai *et al.* 1996). Conversely, little difference in integrin expression in endometriosis has also been described (Bridges *et al.*, 1994; van der Linden *et al.*, 1994). In endometrial adenocarcinomas of varying progression, a complex pattern of integrin expression is described. Notably, the greatest reduction in constitutively expressed epithelial integrin was seen for $\alpha 3$, and the loss of $\alpha 2$ was strongly associated with the existence of lymph node metastasis. Generally, as tumors progressed less integrins were expressed (Lessey *et al.*, 1995a).

The baboon provides a good model of the menstrual cycle and early pregnancy in higher primates (Fazleabas *et al.*, 1997). Here $\alpha 2$, $\alpha 3$ and $\alpha 6$ are constitutively expressed by endometrial epithelial cells. Expression of $\alpha 1$ and $\alpha 4$ is only seen around the time of implantation and is restricted to the epithelium, as seen in human endometrium. These two subunits are lost with the onset of pregnancy in both human (Lessey *et al.*, 1994a) and baboon (Fazleabas *et al.*, 1997). Unlike in

human endometrium, $\alpha v \beta 3$ only appears in baboon endometrial glands following implantation, or in ovariectomised animals following prolonged treatment with oestrogen and progesterone. This correlates with enhanced glandular secretory activity in this species (Fazleabas *et al.*, 1997). Co-expressed with $\alpha v \beta 3$ in baboon early pregnancy was the ECM component OST, suggesting it is a possible ligand for invading trophoblasts (Fazleabas *et al.*, 1997). Secretory phase primate endometrial epithelium is one of the few cell-types expressing $\alpha v \beta 6$ (Breuss *et al.*, 1993, 1995).

The regulation of expression of αv family integrins has also been investigated in mouse endometrium (Aplin *et al.*, 1996) and is described in detail in Chapter 3. Recently it has been reported that in mice, $\alpha v \beta 3$ expression is restricted to the subluminal stroma and is regulated by oestrogen. Apparently intrauterine injection of RGD peptides or a function-blocking antibody against $\alpha v \beta 3$ leads to a reduction in the number of implantation sites (Illera *et al.*, 1997), although this work needs elaboration.

Expression in pre- and peri-implantation stage embryos

Human pre-implantation embryos consistently express the $\alpha 3$, αv , $\beta 1$, $\beta 3$, $\beta 4$ and $\beta 5$ integrin subunits (Campbell *et al.*, 1995b). The expression of various integrin subunits has also been determined in mouse embryos from days 0 to 7.5 of pregnancy. Receptors for fibronectin, laminin and vitronectin ($\alpha 5 \beta 1$, $\alpha 6 \beta 1$ and $\alpha v \beta 3$ respectively) were present throughout this period. $\alpha 2$, $\alpha 6A$ and $\alpha 7$ appear in late blastocysts with the onset of endoderm differentiation and attachment competence. With embryo outgrowth in vitro, $\alpha 1$, $\alpha 6A$ and $\alpha 7$ became trophoblast-specific (Sutherland *et al.*, 1993; Hierck *et al.*, 1993). $\alpha v \beta 3$ is present on the external trophectoderm surface of attachment-competent blastocysts. Furthermore, in trophoblast outgrowths in vitro (on serum proteins), only $\alpha v \beta 3$ is localised to focal adhesions and therefore in the position to directly mediate primary trophoblast adhesion and migration (Sutherland *et al.*, 1993).

Homologous recombination has produced mouse embryos homozygous-null for the $\beta 1$ integrin subunit. These embryos develop normally to the blastocyst stage, attach to the uterine lumen (by day 4.5) and invade through the epithelial basement membrane. Very limited trophoblast invasion of the decidua is seen and ICM cells have degenerated by day 6 of pregnancy (Fassler & Meyer, 1995; Stephens *et al.*, 1995). On FN or VN (but not LM) these embryos form apparently normal trophoblast outgrowths in vitro. The ICM cells of $\beta 1$ -knockout embryos and $\beta 1$ deficient F9 embryonal carcinoma cells exhibit retarded growth and differentiation (Stephens *et al.*, 1995, 1993). Endoderm differentiation is the most affected. $\beta 1$ null embryos do not form a basement membrane between the ICM and endoderm, and as this first basement membrane is required for endoderm survival, it is likely that these cells are continually replaced until the ICM is exhausted (Brakebusch *et al.*, 1997). Embryonic stem cells lacking $\beta 1$ function continue to transcribe integrins $\alpha 3$, $\alpha 5$ and $\alpha 6$, but these are not seen at the cell surface (Fassler *et al.*, 1995). Thus it appears that loss of $\beta 1$ function has direct effects on development of the 'embryo proper', leading to disruption of trophoblast function and failure of early implantation.

The loss of various other integrins has wide-ranging effects, with no disruption of implantation (reviewed by Fassler *et al.*, 1996; Hynes 1996). Animals lacking $\alpha 1$, $\alpha 7$, $\beta 2$, $\beta 6$ or $\beta 7$ are viable. Peri- or early post-natal death occurs in the absence of $\alpha 3$, $\alpha 6$, $\alpha 8$, $\alpha 9$ or $\beta 4$, and pre-natal death occurs without $\alpha 4$ or $\alpha 5$. As αv can bind at least 5 different β subunits it is perhaps surprising that lack of αv function does not manifest until the day of birth, when animals die of vascular hemorrhage (Fassler *et al.*, 1996; Hynes, 1996), further details of which await publication. The effects of loss of $\beta 3$ or $\beta 5$ have yet to be determined. It seems likely that the extensive cross-over between integrin ligands and functions may in part explain the unexpectedly moderate effects seen in integrin knockouts.

Placental integrin expression

In vitro cell-cell attachment assays with carcinoma cell lines have been used to model early implantation. The attachment of placenta-derived JAr cells to

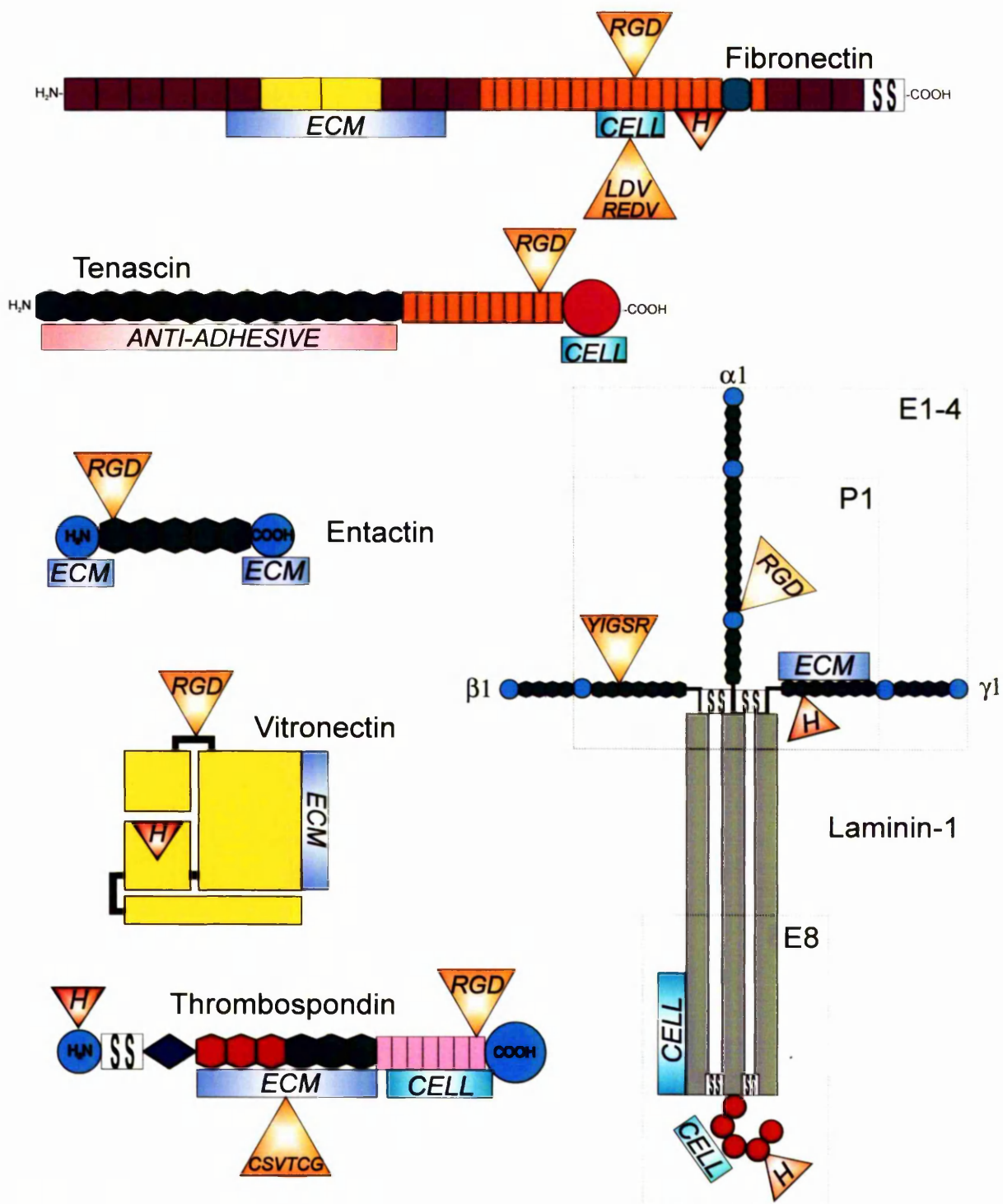
transformed endometrial epithelial cell lines, HEC-1-A (expressing α_v , β_3 and β_5) or RL95-2 (expressing α_v and β_5) showed that RL95 bind twice as many JAr cells as HEC-1-A. Using function blocking antibodies, JAr cell binding to either cell line is reduced with anti- $\alpha_v\beta_5$ but unaffected by anti- $\alpha_v\beta_3$ (Tran & Illsley, 1997).

Tissue samples from early human pregnancy have provided valuable insights into the changing repertoire of integrins expressed by placental CTB populations as they differentiate and invade the uterus (section 1.5.3). CTB stem cells express $\alpha_v\beta_5$ and $\alpha_6\beta_4$. As migratory CTB leave the trophoblast basement membrane, expression of $\alpha_v\beta_5$ is lost. Cells aggregating in columns progressively lose $\alpha_6\beta_4$, $\alpha_v\beta_6$ is transiently expressed and $\alpha_5\beta_1$ appears. Extravillous CTB within the uterine wall retain $\alpha_5\beta_1$ and gain expression of $\alpha_v\beta_3$, $\alpha_1\beta_1$, and $\alpha_4\beta_1$, a pattern which is conserved in CTB invading uterine arteries (Bischof *et al.*, 1993; Damsky *et al.*, 1992, 1994, 1997; Malak & Bell, 1994; Aboagye-Mathiesen *et al.*, 1996). These changes in CTB integrins closely reflect the changes in integrin ligands dominating the extracellular environment (see section 1.8.1; Damsky *et al.*, 1992). Invasive CTB in vitro, when allowed to migrate through a mixed matrix, require $\alpha_1\beta_1$ for interaction with LM and COL IV, and to exhibit the highly invasive phenotype of extravillous CTB (Damsky *et al.*, 1994). Although attachment to LM involves the α_6 subunit (Burrows *et al.*, 1995), $\alpha_5\beta_1$ is necessary to interact with FN via RGD (Burrows *et al.*, 1995), anchoring the less invasive column CTB (Damsky *et al.*, 1994).

1.8 EXTRACELLULAR MATRIX COMPONENTS

For proliferation, migration and invasion cells interact with molecules of the ECM. This 3-D network of fibres usually includes FN, LM, COL, entactin (EN), tenascin (TN) and proteoglycans; in some tissues, thrombospondin (TSP), VN or OST are also present (Hay, 1991). ECM molecules have discrete functional domains, regulating their binding to cells and other matrix molecules. The main structural and functional regions of several ECM components is summarised in Figure 1.4. In this study FN, VN, TSP and Englebreth-Holm-Swarm (EHS) tumour matrix were used as substrates for trophoblast outgrowth in vitro (Chapter 5). Further details of these ECM components are given below.

FN is a dimer (~550kDa) with individual polypeptides linked at the COOH-terminus by two disulphide bonds in an anti-parallel arrangement. FN primarily consists of repeats of three distinct units. Alternatively spliced variants of FN are produced by inclusion of extra type III repeats and/or inclusion of part or all of a type III connecting sequence towards the carboxyl terminus. Inclusion of the type III connecting sequence incurs additional cell-binding activity via REDV and LDV (Ruoslahti, 1988). LM is a heterotrimer consisting of an α , β and γ chain in a cruciform arrangement. First identified was LM-1 (~800kDa), this is a major component of EHS. At least six other LM have since been identified, composed of alternative α , β or γ chains (Timpl & Brown, 1994). TSP is a homotrimeric glycoprotein (~420kDa), the subunits are interlinked by disulphide bonds found close to the amino terminus. At least three TSP exist, encoded by homologous genes (Bornstein, 1992). VN is a major component of serum and also found in the ECM of some tissues. It is a globular protein (~75kDa) that can be proteolytically cleaved, producing a disulphide-bonded (65kDa/10kDa) form. Activation (linearisation) of VN reveals a high affinity heparin binding region (Felding-Habermann & Cheresch, 1993).



STRUCTURAL FEATURES:

- Fibronectin type III repeat
- Procollagen-like repeat
- EGF-like repeat
- Fibrinogen-like domain
- Globular domain
- Disulphide bond

FUNCTIONAL REGIONS:

- Amino acid recognition sequence
- Heparin binding
- ECM binding
- Cell binding
- Anti-adhesion
- Proteolytic fragment

Figure 1.4 Structural features of extracellular matrix components

1.8.1 Expression and function of ECM ligands in implantation

By examining the expression and function of ECM molecules and their receptors in the endometrium and early embryo we can hope to discover the mechanisms by which they facilitate and regulate implantation.

Uterine expression of ECM components

COL I, III and V share a similar distribution in human endometrium, with expression within the stromal ECM and in the blood vessel walls, but not in glands. Levels are highest during the proliferative phase, being markedly reduced during the secretory phase, and persisting in decidualised stroma. COL VI is also most expressed in proliferative phase endometrium, in the stromal ECM and around blood vessels. It becomes less evenly distributed through the stroma during the mid-secretory phase, mainly restricted to blood vessels and glandular basement membrane by the late secretory phase and seen largely around blood vessels in decidua (Aplin *et al.*, 1995, 1988, 1986). COL IV, LM-1 and HSPG are all present in blood vessel walls and in the glandular basement membrane, with expression increasing towards the late secretory phase, in which they are also weakly detected in the stroma. COLs, LMs and HSPG are present in the pericellular basement membrane of decidual stromal cells (Wewer *et al.*, 1985; Aplin *et al.*, 1988). Subunit-specific antibodies have demonstrated hormonally regulated expression of LM-2 and -4 (Church *et al.*, 1996) and expression of isoforms containing the LM α 4 and α 5-chain, by decidual stromal cells (Church *et al.*, 1997). In addition, epithelial and vascular basement membranes are predicted to contain several LM isoforms (Church *et al.*, 1997). Low levels of VN have been described in proliferative phase stroma; these are further reduced in the secretory phase and decidualised stroma. FN is present in the stromal ECM, around blood vessels throughout the menstrual cycle and in first trimester decidua (Aplin *et al.*, 1988). The oncofetal form of FN has been localised to areas of contact between human decidual and trophoblast cells (Feinberg *et al.*, 1991). TSP is localised to the stroma and capillary basement membrane of the functionalis endometrium where its expression is greatly elevated during the secretory phase. It appears to be regulated by progesterone,

an effect mimicked by PDGF in vitro (Iruela-Arispe *et al.*, 1996). OST is expressed in the human uterus where it is localised to the endometrial epithelium, with highest expression during the secretory phase (Brown *et al.*, 1992). Villous CTB also express OST throughout pregnancy (Daiter *et al.*, 1996).

Expression of COL VI in rat endometrial stroma is abundant as in human proliferative endometrium. On artificial induction of a decidual response COL VI disappears within 72 hours, and returns as the response regresses (Mulholland *et al.*, 1992). COL IV and LM γ 1-chain mRNA are at high levels in the primary and secondary zones of decidualisation in the mouse uterus. LM β 1-chain is found at higher levels than LM γ 1-chain and restricted to the primary decidualised zone (Farrar & Carson, 1992; Glasser *et al.*, 1987). LM and COL IV of the uterine epithelial basement membrane are lost as the blastocyst invades through the epithelium and breaches the basement membrane (Blankenship & Given, 1995). Two splice variants of FN are expressed in the rat uterus and are highest in decidualised stroma on day 6 of pregnancy, where staining is restricted to the subluminal stroma of the mesometrial zone (opposite the site of implantation; Rider *et al.*, 1992). In mouse decidua, FN has been shown to be differentially organised by distinct stromal subpopulations in vitro (Babiarz *et al.*, 1996). During pregnancy in mice, TSP is localised to endometrial glands and decidua (Corless *et al.*, 1992). In the mouse, mRNA encoding OST was detected in many tissues including the uterus, where it is increased in pregnancy, and placenta, and is upregulated by progesterone (Craig & Denhardt, 1991). Deposition of TN is greatly increased during the peri-implantation period in the mouse uterus. TN deposits accumulate in the stromal ECM immediately adjacent to site of embryo attachment and may disrupt adhesion of the uterine epithelium to the underlying basal lamina, thus facilitating invasion (Julian *et al.*, 1994). In addition, moderate levels of VN mRNA have been detected in the murine uterus (Seiffert *et al.*, 1994).

ECM molecules in pre-implantation embryos and invasive trophoblast

The oncofetal variant of FN is expressed by human pre-implantation embryos (Turpeenniemi-Hujanen *et al.*, 1995). LM is also present in human embryos from the morula stage (Turpeenniemi-Hujanen *et al.*, 1992). The changing ECM which surrounds CTB as they differentiate and migrate through the uterus is reflected by the integrins they express (section 1.7.6). The trophoblast basement membrane contains LM and COL IV. Through the CTB column LM is reduced, and FN, VN and HSPG appear (Frank *et al.*, 1994). Within the decidual ECM, LM again is a major component (Damsky *et al.*, 1992). OST expression in the placenta is restricted to villous CTB (Omigbodun *et al.*, 1995), present throughout pregnancy (Daiter *et al.*, 1996) and regulated by progesterone (Omigbodun *et al.*, 1997).

In the mouse, expression of TSP is localised to the cytoplasm of unfertilised eggs and in embryos up to 4-cell stage. By the 8-cell stage TSP is deposited at cell borders, and in blastocysts it is present in the ICM and at the trophectoderm surface (O'Shea *et al.*, 1990). LM and EN are found in peri-implantation stage blastocysts, localised to the apical trophoblast (Wu *et al.*, 1983; Dziadek & Timpl, 1985; Carson *et al.*, 1993). Also LM is a component of the first basement membrane formed during mouse development (Hierck *et al.*, 1993). HSPG is expressed at the apical trophoblast surface at the time in which mouse blastocysts become attachment competent both in vitro and in vivo, and levels decrease with embryo outgrowth (section 1.6.1; Farach *et al.*, 1987).

1.8.2 In vitro trophoblast outgrowth on ECM substrates

In vitro models of trophoblast outgrowth on three-dimensional matrices, single substrate-coated surfaces, and interaction between human placental and uterine cells in vitro have provided insights into the mechanism of embryo attachment and early invasion.

ECM purified from mouse endometrium on day 4 of pregnancy provides a mixed three-dimensional substrate to which mouse blastocysts will attach (via the

abembryonic pole) and invade (Armant & Kameda, 1994). Chondroitin sulphate proteoglycans (CSPG) are the major class of proteoglycan synthesised by mouse uterine stromal cells *in vitro*. Outgrowth on purified stromal ECM is enhanced if the matrix is pre-treated with chondroitinase, suggesting that CSPG can modulate trophoblast invasion (Carson *et al.*, 1992b). When placed on EHS matrix, marmoset blastocysts attach via their embryonic pole within 24 hours and subsequently invade the matrix. The phenotype of invading trophoblast populations reflects that seen *in utero* (Lopata *et al.*, 1995).

Mouse blastocysts outgrow on a FN substrate in an RGD-dependent manner (Armant *et al.*, 1986a, b), mediated by a 120kDa fragment of FN and independent of heparin (Yelian *et al.*, 1995). This outgrowth can be inhibited using function-blocking antibodies to $\beta 1$ or $\beta 3$ integrins (Yelian *et al.*, 1995). Beads coated with the 120kDa cell-binding fragment of FN (FN-120) bind to the abembryonic pole of mouse blastocysts in an GRGDSP-peptide-dependent manner (Schultz & Armant, 1995). Soluble laminin does not block bead binding, supporting earlier suggestions of different receptors (Armant *et al.*, 1986b, 1991; Yelian *et al.*, 1993). Binding of FN beads to mouse blastocysts requires divalent cations (10mM Ca^{2+} or Mn^{2+} ions, or 0.01mM Mg^{2+}) suggesting an integrin-mediated interaction. Anti-integrin function-blocking antibodies to $\alpha 5$ or $\beta 1$ subunits reduced bead-binding by 65% and anti- αv or $\beta 3$ reduced bead-binding by 35%. To acquire this FN-binding activity, a pre-requisite for outgrowth, blastocysts require potentiation (pre-exposure to ligand). Potentiation is ATP-dependent and requires an intact cytoskeleton and membrane transport processes, suggesting receptor recruitment and relocation, rather than *de novo* DNA transcription and protein synthesis (Schindler & Sherman, 1981; Schultz & Armant, 1995). Thus it seems that interaction of the apical trophectoderm with endometrial ECM mobilises stores of accumulated gene products required for trophoblast invasion.

The RGD recognition sequence has also been demonstrated, at least in part, as mediating outgrowth on LM, EN, COL II and COL IV (Sutherland *et al.*, 1993;

Yelian *et al.*, 1993; Carson *et al.*, 1988). VN also supports outgrowth; this is expected to be RGD-dependent (Armant *et al.*, 1986b). When coverslips are precoated with the hexapeptide GRGDSP embryo outgrowth is supported but outward migration is limited, indicating a multistep process (Armant *et al.*, 1986b). It has been reported that blastocysts outgrow on TSP and that this is independent of GRDGs (O'Shea *et al.*, 1990).

As LMs are a major component of decidual ECM (section 1.8.1) the mechanism by which LM supports outgrowth has been extensively investigated. The ability of LM to support outgrowth is dependent on the protein backbone of the molecule and does not require the carbohydrate moieties it displays (Armant, 1991). Mouse LM-1 can be proteolytically cleaved into at least three fragments (see Figure 1.8.1), releasing primarily the 'E8' and 'E1-4' fragments. LM-E1-4 contains a cryptic RGD sequence (within the P1 sub-fragment) and YIGSR, both of which can be recognised by cells (Timpl & Brown, 1994). Armant *et al.* (1986b) showed that outgrowth on intact LM is GRGDSP-independent. Although LM-P1 supports outgrowth via GRGDSP (Sutherland *et al.*, 1993), intact LM-E1-4 fragments or CDPGYIGSR do not (Armant *et al.*, 1991). The LM-E8 fragment supports outgrowth independent of its heparin-binding activity or RGD (Armant *et al.*, 1991; Sutherland *et al.*, 1993). This fragment contains IKVAV, a cell recognition sequence with trophoblast binding activity (Romagnano & Babiarz, 1993). Thus it appears that outgrowth on intact LM is mediated by a cell binding region of LM-E8, possibly mediated by $\beta 1$ family integrins (independent of RGD).

Armant *et al.* (1986b) suggested that COL I, COL III and COL IV do not support attachment and outgrowth of blastocysts (by 72 hours). However other studies have demonstrated that embryos can outgrow on COLs I-VI (Carson *et al.*, 1988; Sutherland *et al.*, 1988). The ability to outgrow on COLs II and VI was acquired early on whereas outgrowth on other COLs was more delayed (seen by 120 hours). Cell attachment to COL was independent of heparin or heparan sulphate and an ability to outgrow on all but COL V was retained following heat denaturation (Carson *et al.*, 1988).

The rate of embryo outgrowth on FN, LM and uterine epithelial monolayers decreases in the presence of heparin in the culture medium (Farach *et al.*, 1987), suggesting that HSPG on the apical trophectoderm surface (Carson *et al.*, 1993) may also mediate embryo attachment (section 1.6.1) and outgrowth on ECM substrates. Alternatively this interaction may be more important at the attachment phase of embryo outgrowth (section 1.6.1; Carson *et al.*, 1990b). HSPG is also a substrate for embryo outgrowth, mediated by the polysaccharides of HSPG rather than the protein core (Farach *et al.*, 1987, 1988).

Isolated human trophoblast cells can adhere to FN (Burrows *et al.*, 1993) and LM (Loke *et al.*, 1989) in vitro. Human decidua monolayers continue to produce ECM in vitro, and isolated trophoblasts adhere strongly to these cells and their matrix (Burrows *et al.*, 1997). Furthermore, outgrowth but not attachment of mouse blastocysts to human decidual cell monolayers is significantly inhibited by function-blocking antibodies to $\beta 1$ family integrins, possibly via $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$ or $\alpha 6\beta 1$ (Shiokawa *et al.*, 1996b).

1.9 AIMS OF THE STUDY

Several mechanisms have been implicated in complex adhesive events that occur at implantation. The present study aimed to investigate the role of integrins, focusing on a subgroup which contain the α_v subunit ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$). Wherever possible comparisons were made between human and mouse. A broad approach was taken, examining α_v integrins in both endometrial and embryonic tissues, and the role of these during the attachment and invasion phases of implantation.

Endometrial samples from human and mouse were examined for the expression and localisation of α_v integrins. Subunits identified at the uterine epithelium may play a role in embryo attachment; integrins expressed in the stroma may be important later in implantation, during trophoblast invasion. Endometrial tissue of known hormonal status allowed the control of integrin expression to be studied.

Human and mouse blastocysts were used to determine which α_v integrins might be important at embryo attachment. An in vitro model of mouse implantation, in which trophoblast cells outgrow on serum components, allowed the localisation of trophoblasts α_v integrins during the invasion stages of implantation.

Addition of function-blocking reagents to the in vitro model of mouse implantation allowed the role of α_v and β_1 integrin subgroups to be determined during development of trophoblast outgrowths, on defined substrates known to be present in the endometrial ECM. This allowed examination of the relative importance of different integrin ligands; including those commonly found within the basement membrane of the uterine epithelium or throughout the endometrial stroma, some of which are upregulated around the time of implantation. Anti-integrin antibodies shown to inhibit trophoblast outgrowth on defined substrates in vitro were used to investigate the role of integrins in mouse implantation in utero. In the absence of the appropriate reagents, new function-blocking antibodies would be raised to allow further functional studies of individual integrins (of the α_v subgroup) in implantation.

CHAPTER 2

Materials and methods

2.1 - INTEGRIN EXPRESSION AND FUNCTION IN TISSUES

2.1.1 Human endometrium

Human endometrial tissue was obtained as a result of dilatation and curettage operations. First trimester decidua samples were obtained by dissection from the products of elective pregnancy terminations. In all cases human tissue was obtained with patient consent and under local ethical committee approval. Tissue was embedded in OCT compound (R. Lamb, Cambridge), snap frozen in liquid nitrogen and stored at -80°C until required. Transverse sections of 6µm were cut using a Reichert-Jung cryostat. Sections were air dried and stored at -20°C until use. Human tissue was dated using the criteria of Noyes *et al.* (1950) following histological examination by pathologists at St Mary's Hospital (Manchester), which allowed exclusion of neoplastic and infected samples from the study.

2.1.2 Mouse endometrium collection

MF1 mice (Harlan OLAC, Bicester, UK) were kept in a controlled environment with a cyclic photoperiod of 12 hours light followed by 12 hours darkness and provided with food and water ad libitum. Tissue was collected from female mice which were either non-pregnant, pregnant (naturally mated) or which had undergone ovariectomy and steroid hormone replacement. Non-pregnant mice were staged with respect to the estrus cycle by daily examination of vaginal flushings under a light microscope and classed as pro-estrus, estrus, metestrus and diestrus. For natural matings females were paired with male mice and left until mating could be confirmed by the presence of a vaginal plug, this day was designated as day 1 of pregnancy. Hormonally treated mouse uterus was kindly provided by Ian Illingworth, University of Manchester. Six week old mice were ovariectomized under hypnorm anesthetic, rested for 10 days then injected with vehicle (corn oil) or 100ng oestradiol benzoate daily for 2 days followed by 2 days rest. Oestrogen-primed animals were then divided into three groups. The first group received 100ng oestradiol benzoate daily for a further 4 days. The second group received 500µg progesterone daily for 4 days. The third group received 500µg progesterone daily for 3 days then 500µg progesterone with 10ng oestradiol benzoate on the fourth day. Ovariectomized animals were sacrificed 16 to 18 hours after the last injection.

Animals were killed by cervical dislocation (Schedule 1, Scientific Procedures Act 1986, HMSO). The abdomen of the animal was swabbed with 70% ethanol and the peritoneal cavity was opened. Uterine horns were trimmed of fat, removed and placed in Hank's Balanced Salt Solution (HBSS; Gibco BRL). Tissue to be used for immunolocalization studies was cut transversely into 5mm pieces, embedded in OCT compound, and frozen and sectioned as described above (2.1.1).

2.1.3 Immunocytochemistry of endometrial tissue

Cryosections were fixed in acetone for 10 minutes at 4°C then brought to room temperature. After thorough washing in phosphate buffered saline (PBS, Appendix 1), sections were incubated with normal goat serum (Sigma) diluted 1:20 in PBS, for 30 min to prevent non-specific binding. They were then incubated in 50µl to 100µl primary antibody (Table 2.1) for 60 min in a humid chamber. After further washes sections were incubated in the appropriate biotin-conjugated secondary antibody (Table 2.2) for 60 min. Once washed, sections were incubated in fluorescein isothiocyanate (FITC)-conjugated streptavidin for 60 min (1:50; Dakopatts). Sections were again thoroughly washed in PBS then mounted in Immu-Mount (Shandon). Controls using non-immune rabbit serum or PBS in place of primary antibody, secondary antibody or FITC-streptavidin were performed. Slides were viewed under epifluorescence and photographed using HP5 film (Ilford).

2.1.4 Human embryo acquisition

Human embryos donated for research by patients at Leeds General Infirmary with full ethical approval were maintained in vitro to the blastocyst stage in the laboratories of Dr. Henry Leese, University of York. Blastocysts were fixed in 2% paraformaldehyde at day 6 or 7 of development and sent to our laboratory. Prior to receipt, embryos had been used in non-invasive metabolic studies, cultured in medium with either high or low amino acid concentration. On receipt, blastocysts from different culture environments were first pooled and then randomly split into groups for staining with different antibodies (2.1.5).

Table 2.1 Primary antibodies

| Antigen (species) | Antibody (fraction) | Working dilution | Code, supplier (reference) |
|--|--------------------------------|--|---|
| α v integrin (human) | Rab. poly. | 1:50 ^a 1:100 ^b | AB1930, Chemicon International (Ruoslahti & Pierschbacher, 1987) |
| α v integrin (human) | Ms. mab (IgG) | 1:100 ^a 1:500 ^b | MAB1978, Chemicon International (Cheresh, 1987) |
| α v integrin (mouse) | Rat mab. (IgG) | 1:100 ^a 1:10 ^d | C8F12, Charles Streuli, Manchester University |
| α 4 β 1 integrin (mouse) | Rab. poly. (IgG) | 1:50 ^a 1:20 ^d 1:100 ^b | R48, C Streuli, Manchester Univ. (Pullan <i>et al.</i> , 1996) |
| β 1 integrin (human) | Ms. mab (IgG) | 1:100 ^a | MCA780, Serotec (Ylanne <i>et al.</i> , 1990) |
| β 3 integrin (human) | Rab. poly. | 1:50 ^a 1:100 ^b | AB1932, Chemicon International (Ruoslahti & Pierschbacher, 1987) |
| β 5 integrin (human) | Rab. poly. | 1:50 ^a 1:100 ^b 1:100 ^c | AB1926, Chemicon International (Smith <i>et al.</i> , 1990) |
| α v β 5 integrin (human) | Ms. mab (IgG) | 1:200 ^b | P1F6, Chemicon International (Wayner <i>et al.</i> , 1991) |
| FN (human) | Rab. poly. | 1:100 ^c | AB1940, Chemicon International (Beardsley <i>et al.</i> , 1983) |
| TSP (human) | Rab. poly. | 1:100 ^d | RAB35-650 Scottish Antibody Production Unit |
| GST (bovine) | Rab. poly. | 1:1000 ^c | 27-4577-01, Pharmacia Biotech (Kaelin <i>et al.</i> , 1991) |

^a Immunocytochemistry of embryos/outgrowths^b Immunocytochemistry of endometrial tissues^c Chemiluminescent detection^d Outgrowth blocking studies

Rab. poly. - rabbit polyclonal

Ms/Rat. mab. - mouse/rat monoclonal

Table 2.2 Secondary antibodies

| Antigen (species) | Conjugate | Working dilution | Supplier (clone) |
|------------------------------|------------------|-----------------------------|-----------------------------|
| IgG (rabbit) | FITC | 1:50 ^a | Sigma (F1262) |
| IgG (rabbit) | Biotin | 1:50 ^b | Dakopatts (E431) |
| IgG (rabbit) | Peroxidase | 1:1000 ^c | Sigma (A4914) |
| IgG (mouse) | FITC | 1:50 ^a | Sigma (F9006) |
| IgG (mouse) | Biotin | 1:50 ^b | Dakopatts (E0354) |
| IgG (rat) | FITC | 1:50 ^a | Sigma (F6258) |
| IgG (goat) | Peroxidase | 1:1000 ^c | Sigma (A4174) |

^a Immunocytochemistry of embryos/outgrowths

^b Immunocytochemistry of endometrial tissues

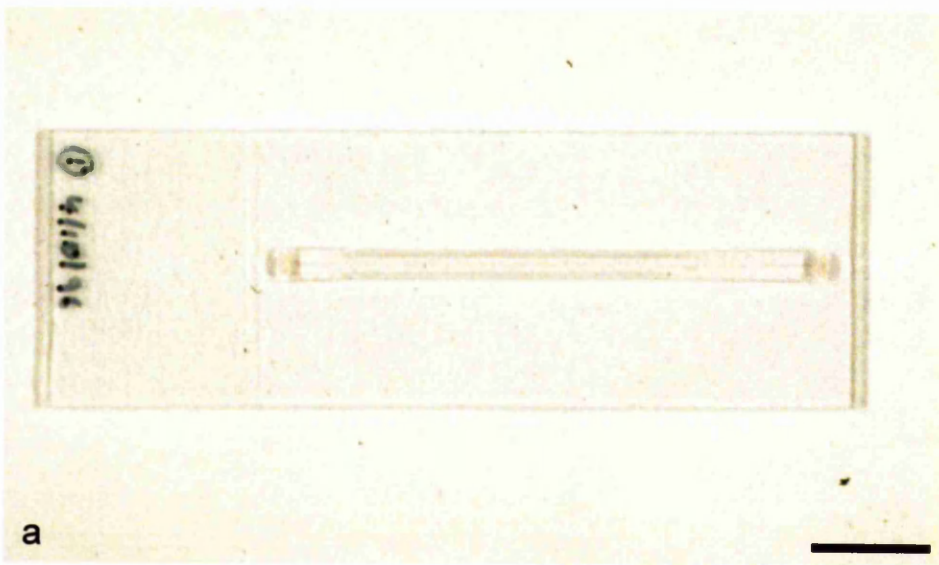
^c Chemiluminescent detection

2.1.5 Immunocytochemistry of human blastocysts

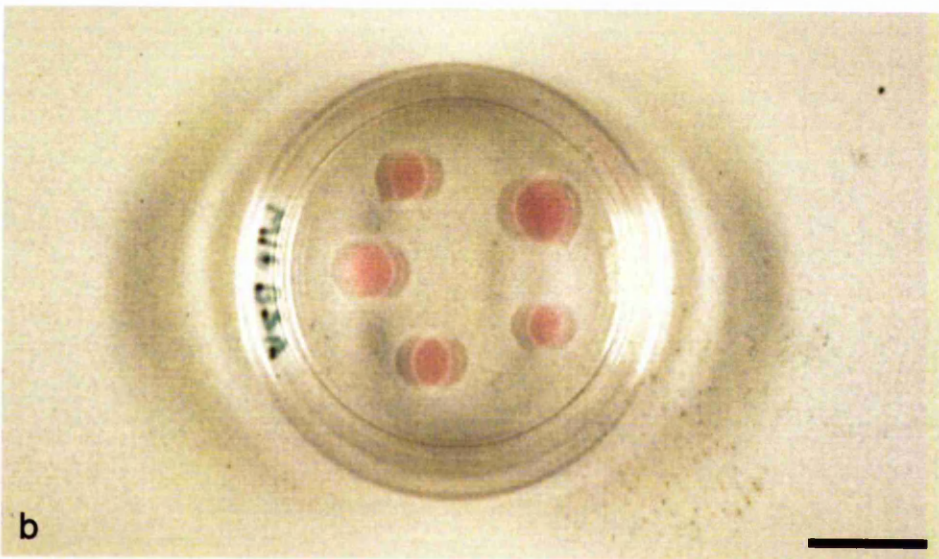
For staining using antibodies directed to the cytoplasmic tail of the integrin subunits, embryos were re-fixed in 1% paraformaldehyde (PFA; in PBS) for 10 min, washed through four drops of PBS containing bovine serum albumin (BSA, 4mg/ml; termed 'PBS wash solution') permeabilized in 0.1% Triton X-100 (in PBS wash solution) for 2 min and washed as above. For antibodies to the extracellular domain of integrins, embryos were washed as above after receipt. Subsequently all embryos were stained using the protocol described below.

Drops of primary antibody diluted in PBS wash solution (25 μ l) were prepared in a 35mm culture dish and covered with paraffin oil. Embryos were transferred to these drops and incubated at room temperature for 90 min. They were washed by passing quickly through several drops of PBS wash solution, incubated in a fresh wash drop under oil for 60 min and then moved to a final wash drop under oil and placed at 4°C over night. Embryos were then incubated in drops of secondary antibody diluted in PBS wash solution (25 μ l) under oil at room temperature, in the dark for 90 min. Embryos were extensively washed overnight as described above. Finally embryos were transferred to PBS wash solution containing DABCO (1, 4 diazobicyclo-(2, 2, 2) octane; Boehringer Mannheim; 12.5mg/ml) and drawn up a 0.2mm microslide (Camlab, Cambridge) and mounted onto a glass slide (2.1.7) as shown in Figure 2.1a. Slides were then examined immediately by confocal microscopy, as described further in section 2.1.14 following identification of blastocysts under UV illumination.

Mounted microslide - embryo staining



Embryo culture drops



Serum-free outgrowth culture

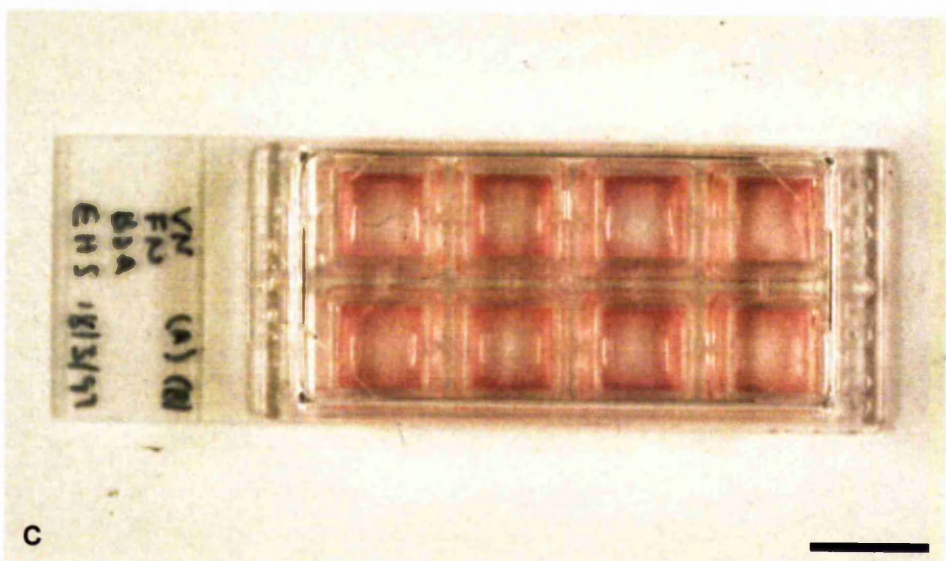


Figure 2.1 Apparatus used in embryo studies

Scale bar = 1cm

2.1.6 Mouse embryo collection and culture

2.1.6.1 Superovulation

Immature female mice (20-24 day old) underwent a superovulatory regime to maximize oocyte yield. Intraperitoneal injection of 5IU Pregnant Mares Serum Gonadotrophin (PMSG; Intervet) was followed, 44 to 48 hours later, by 5IU Human Chorionic Gonadotrophin (HCG; Intervet); under Home Office License. The animals were then paired with MF1 male mice and left overnight. Mating was assumed to occur at approximately midnight and was confirmed by the presence of a vaginal plug. On day 2 of pregnancy, 35 hours post-mating, the animals were killed. Oviducts were carefully dissected out and placed in sterile Ham's F10 medium (Gibco BRL) containing 4 mg/ml BSA (ICN Biomedicals; termed 'flushing medium').

2.1.6.2 Oviduct flushing

Embryos were flushed from oviducts under sterile conditions, visualized using a Wild dissecting microscope. A 30 gauge blunt-end needle attached to a 1 ml syringe containing flushing medium was inserted into the fimbriated end of the oviduct. Approximately 0.1ml of flushing medium was injected to "flush" each oviduct, this was done within a drop of flushing medium. Embryos were washed of any debris by passing through four successive drops of flushing medium in the lid of a sterile 60mm culture dish (NUNC) and were separated from embryos of abnormal morphology or development, so that only apparently healthy two, three or four-cell embryos were retained. Manipulations were carried out using a mouth-pipette made from a flame-pulled sterile Pasteur pipette (Liquid Equipment and Services Ltd.) attached via rubber tubing to a mouth-piece.

2.1.6.3 Embryo culture

Once washed, embryos were passed through two drops of pre-incubated M16 medium (Sigma) containing 4mg/ml BSA and penicillin/streptomycin (Sigma, at 60µg/ml and 50µg/ml respectively; termed 'embryo medium') and transferred to culture drops. Embryos were cultured in groups of up to 400 in approximately 25µl drops of embryo medium in a 35mm culture dish (NUNC) covered with paraffin oil (BRL). Culture drops were prepared prior to flushing and incubated to

allow equilibration (Figure 2.1b). Embryos were maintained at 37°C with 5% CO₂. At this stage embryos were termed Day 2 of development. The various stages of embryo development seen during this culture period are described in Chapter 4.

2.1.7 Immunocytochemistry of murine embryos

Embryos were taken from the culture drop on days 4, 5 and 6 of development using a mouth-pipette. They were washed through three drops of M2 medium (Quinn *et al.*, 1982) containing BSA (4mg/ml) and sodium azide (at 0.02%, to prevent antigen capping; termed 'M2 wash solution') in the lid of a 60mm culture dish. Unhatched embryos were treated with acid Tyrode's solution as described in section 2.1.8, washed in M2 wash solution and combined with any naturally hatched blastocysts.

For staining with antibodies directed against the cytoplasmic tail of integrin subunits, embryos were fixed in 0.1% PFA in PBS for 10 min at 4°C, in a solid watch glass. Embryos were then washed in four drops of PBS wash solution and permeabilized in a drop of 0.01% Triton-X100 (Sigma), in a solid watch glass, for 1 min at 4°C and again washed in PBS wash solution. All subsequent washes and incubations of fixed embryos were done in PBS wash solution. For antibodies (Table 2.1) to the extracellular domain of integrins, embryos were not fixed or permeabilized and were therefore live. All washes and incubations of live embryos were carried out in M2 wash solution.

To adsorb out antibodies to keratin from the rabbit antiserum, human keratin (8.3mg/ml; Sigma) was added to neat rabbit antiserum at a ratio of 1:2 and this was then diluted with the appropriate wash solution to produce the desired final concentration of antibody solution (Table 2.1). The solution was left for 30 min and then centrifuged at 3000g for 5 min to precipitate any keratin-antibody complexes. The supernatant was then used as the primary antibody solution for staining embryos.

Drops of approximately 35µl pre-adsorbed primary antibody and control solutions (pre-immune antiserum or PBS wash solution) were placed into labeled culture

dishes and covered with paraffin oil. Embryos were transferred to the drops (in groups of approximately 30) and left at room temperature for 60 min. The secondary antibody solution (Table 2.2) was prepared by dilution in wash solution; this was centrifuged at 3000g for 10 min to remove precipitates. After five washes, embryos were incubated in secondary antibody drops under paraffin oil for 60 min in the dark. Finally, fixed embryos were washed in five drops of PBS wash solution containing 25mg/ml DABCO to prevent deterioration of the fluorescence (Johnson *et al.*, 1982). Unfixed embryos were washed in five drops of M2 wash solution without DABCO, as this is toxic to pre-implantation embryos.

Embryos in the final wash drop were drawn up into a microslide (path length 0.1mm, or 0.2mm for expanded blastocysts; Camlab) by capillary action. The ends of the microslide were plugged with plasticine and the microslides were mounted on glass slides using superglue (Figure 2.1a). Embryos were viewed immediately on a Leitz epifluorescent microscope and photographed using TMAX 400 black and white film.

2.1.8 Mouse embryo outgrowth in vitro in serum-containing medium

A published method (Sutherland *et al.*, 1988) for producing trophoblastic outgrowths in medium containing fetal calf serum (FCS, Gibco BRL) was optimized. Day 6 embryos at the blastocyst stage were used in outgrowth experiments, the first day of outgrowth culture is termed day 1 of outgrowth. Unhatched blastocysts were transferred, in groups of not more than 50, to a drop of 0.1% acid Tyrode's solution (pH 2.3) for approximately 20 sec, for chemical removal of the zona pellucida, washed in four drops of embryo medium and mixed with naturally hatched blastocysts. Blastocysts were then transferred in groups of 5 to 4-well multidishes (NUNC), where each well contained a clean sterile 13mm glass coverslip (Chance Proper Ltd.) and 500 μ l of filter sterilized (45 μ m pore size, low protein binding; Gelman) embryo medium (2.1.6) containing 10% heat inactivated FCS, this is termed day 1 of outgrowth. Embryos were cultured in serum containing medium for up to 6 days, at 37°C in an atmosphere of 5% CO₂.

2.1.9 Immunocytochemistry of mouse embryo outgrowths

Trophoblast outgrowths on glass coverslips were maintained in serum containing cultures (2.1.8) for 2 to 7 days and were fixed at various stages with 1% paraformaldehyde in PBS for 10 min at room temperature. Outgrowths were washed three times for 5 min in PBS containing 1 mg/ml BSA, 0.5mg/ml Tween-20 (Biorad) and 0.2mg/ml sodium azide (BDH), termed 'outgrowth wash' and permeabilized with 0.1% Triton X-100 in PBS for 5 min then washed again as above.

Coverslips were removed from the wells and the outgrowths were blocked by adding 100 μ l of 1:20 normal goat serum (Sigma) in outgrowth wash to the top of each coverslip. After 30 min the blocking solution was drained off the coverslips and replaced with 100 μ l of primary antibody (Table 2.1) pre-adsorbed with human keratin (2.1.7) and diluted in outgrowth wash. After incubation at room temperature for 60 min antibody solution was drained off and coverslips were washed as described above. Secondary antibody (Table 2.2) was prepared by dilution in outgrowth wash and centrifugation at 3,000g for 10 min to remove precipitates, then added to the coverslips which were then kept in the dark for 60 min. Finally coverslips were washed as above with outgrowth wash containing DABCO (12.5mg/ml) and mounted by inverting onto a drop of Gelvatol non-fade mountant on glass slides (Appendix 1). Slides were then viewed on a confocal microscope, as described in section 2.1.14 following identification of outgrowths under bright field illumination.

Trophoblast outgrowths cultured in serum-supplemented medium, carried out in the presence of low levels (1:100) of anti-integrin antibodies (2.1.11) were removed from culture when appropriate, briefly washed in outgrowth wash, incubated in secondary antibody (Table 2.2), washed again and mounted as described above.

2.1.10 Mouse embryo outgrowth in vitro in serum-free medium

A method for producing outgrowths on specific substrates under serum-free conditions (Armant *et al.*, 1986b) was adapted to allow examination of the role of specific integrin subunits in trophoblast outgrowth.

2.1.10.1 Substrate coating

Wells of an 8-chamber glass slide (NUNC; Figure 2.1c) were pre-coated with 100µl of the appropriate substrate. FN, VN, TSP (Sigma) EHS matrix (courtesy of Charles Streuli, University of Manchester) were prepared in ice cold HBSS (containing 1mM calcium chloride for use with TSP; Lawler *et al.*, 1988) at 25 µg/ml and allowed to adsorb to the glass by incubation at 37°C for 60 min. Alternatively 100µl of HBSS containing 10% heat inactivated FCS was placed in the well and incubated as above. The substrate solutions were then removed and replaced with HBSS containing heat inactivated BSA (10mg/ml) and incubated at 37°C for 60 min to block non-specific outgrowth of embryos (wells pre-coated with BSA alone served as controls).

2.1.10.2 Serum-free outgrowth culture

CMRL1066 medium (Gibco BRL) supplemented with 0.5mg/ml calcium lactate (Sigma), 0.05mg/ml sodium pyruvate (Sigma), 4mg/ml BSA and penicillin-streptomycin (60µg/ml and 50µg/ml respectively) was prepared and syringe filtered (0.22µm pore size, low protein binding; Millipore) prior to the addition of 1% Nutridoma-HU (Boehringer Mannheim). Hatched blastocysts were transferred in groups of 10 to 20 into chambers of an 8-well glass slide (NUNC; Figure 2.1c) containing 100µl of pre-incubated culture medium, this is termed day 1 of outgrowth. Under serum-free conditions outgrowths were maintained for up to 4 days.

2.1.10.3 Confirmation of thrombospondin coating

As no embryo outgrowth was observed on TSP an immunological detection method was used to demonstrate coating of the glass slide with this substrate. For this, wells were uncoated (negative control) or coated in either TSP or FN (positive control) and blocked with BSA as described above (2.1.10.1). Primary

antibodies (rabbit polyclonal, see Table 2.1) to TSP and FN were added to the uncoated wells and those coated with TSP and FN respectively. After incubation for 60 min the antibody was removed and the wells were washed three times for 5 min with (PBS). Wells were incubated for 60 min in an anti-rabbit peroxidase-conjugated secondary antibody (Table 2.2) and then washed as above. Immunoreactivity was detected using the ECL chemiluminescent reagent (Amersham Life Sciences) as described further section 2.3.13.

2.1.11 Addition of function blocking antibodies to outgrowth cultures

Outgrowths in media with or without serum were also carried out in the presence of function blocking antibodies (Table 2.1) directed to the α_v (C8F12; section 2.1.13) or β_1 (R48) integrin subunits. For these experiments an appropriate volume of filter sterilized antibody was added to the culture medium on day 1 of outgrowth, where outgrowths were set up as described in sections 2.1.5 and 2.1.6.

2.1.11.1 Determination of antibody dose required

Outgrowths cultured in serum-free medium (2.1.10) were used to determine the concentration at which the α_v and β_1 antibodies can block trophoblast outgrowth on serum and EHS substrates respectively. Antibodies were diluted in culture medium to 1:5, 1:10, 1:20 and 1:100 to determine at which concentration they have a marked effect on outgrowth and then the appropriate dilution (1:10 for α_v , 1:20 for β_1 or both antibodies) was used in subsequent outgrowth (serum-free) blocking studies.

2.1.11.2 Outgrowth analysis

Purified rat IgG or no antibody was used in place of the α_v or β_1 antibody respectively to serve as controls. The numbers of embryos attached to or outgrowing on the various substrates in the presence of antibodies (or control substances) were counted on days 3 and 4 of outgrowth. The existence of trophoblast cells outgrowing from the main mass of the embryo was determined by examination of each embryo at X10 and X20 magnification. Data was statistically analyzed using a chi-squared test. Some outgrowths cultured in

serum containing medium had the lowest level (1:100) of αv or $\beta 1$ antibody (Table 2.1) added. At this concentration no effect on the extent of trophoblast outgrowth was seen in any embryos. These outgrowths were used in immunocytochemistry studies described in section 2.1.9

2.1.12 Injection of an anti- αv antibody into mouse uterus in vivo

In preliminary experiments to examine the feasibility of in vivo studies of integrin function, 6 week old naturally mated mice underwent intra-uterine injection of an anti- αv antibody (C8F12) or control substances. At 2.30pm to 5.30pm on day 4 of pregnancy (86.5 to 89.5 hours post-mating) animals were anesthetized by intra-peritoneal administration of a 1:1 solution of hypnorm (1:1 in sterile water) and midazolam (1:1 in sterile water) at approximately 7 μ l/g body weight. Bilateral dorsolateral incisions of 5mm were made on each animal to allowing access to the ovary and uterine horn. The ovary was carefully exposed by manipulation of the ovarian fat pad and the number of corpus lutea on each ovary were recorded.

Unilaterally the oviduct-end of the uterine horn was carefully injected with 20 μ l of sterile antibody (500 μ g/ml in sterile saline) or control substance (rat IgG (500 μ g/ml; Sigma) or saline through the uterine wall into the lumen. For this a 100 μ l Hamilton syringe (Howe) was used. The substance was slowly released into the uterine lumen to minimize tissue disturbance. Once the intra-uterine injection was complete the uterus and ovary were gently returned to the peritoneal cavity by manipulation of the ovarian fat pad; the whole procedure took about 20 min per animal. The mice were returned to their cage which was left on a 37°C heated pad overnight. On day 6 or 7 of pregnancy animals were killed and the numbers of implantation sites were counted.

2.1.13 Purification of an anti- αv antibody from the C8F12 hybridoma clone

2.1.13.1 Maintenance of the C8F12 cell line

A rat lymphocyte hybridoma cell line (clone C8F12) producing an antibody raised to the mouse αv integrin subunit was a kind gift of Charles Streuli (University of Manchester). The cells were maintained in 75cm² flasks in 10ml of serum free hybridoma medium (Appendix 1) at 37°C and passaged twice weekly. When

confluent cells were dislodged by moderate agitation of the culture flask and split (1:4). Cell-conditioned medium was collected for purification of secreted antibody, centrifuged at 1,000g for 10 min at 4°C to pellet cell debris and the resulting supernatant was stored at -20°C until required.

2.1.13.2 Purification of hybridoma supernatant

Antibody was purified from 500ml of conditioned medium on a protein G Sepharose column (by Gary Broomhead, University of Manchester). Briefly, a 10ml protein G Sepharose column was poured and allowed to equilibrate in 0.1M Tris buffer (pH 8). The pH of the conditioned medium was adjusted to pH 8 using 1M Tris buffer (pH 8). Overnight the conditioned medium was passed through the protein G Sepharose column. The column was washed, first with 100ml of 0.1M Tris buffer (pH 8) followed by 100ml of 10mM Tris buffer (pH 8). Bound antibody was eluted from the column in 0.1M glycine (pH 3) and fractions were collected in tubes containing 1ml of 1M Tris buffer (pH 8). The various fraction were measured at 280nm and samples with an absorbance greater than 0.1 were pooled (approximately 10ml). Protein concentration was calculated as follows (based on $A_{280\text{nm}} = 1$ at 0.75mg/ml):

$$[\text{IgG}] = A_{280\text{nm}} \times \text{constant}$$

For example,

$$\begin{aligned} [\text{IgG}] &= 0.359 A_{280\text{nm}} \times 0.75\text{mg/ml} \\ &= 0.27\text{mg/ml} \end{aligned}$$

To remove glycine the antibody fraction was dialyzed twice against 5L of PBS overnight and then against 5L of distilled water overnight. The resulting antibody solution was concentrated in a freeze drier (Genevac) to a volume of approximately 1.5ml, giving a final [IgG] of approximately 2mg/ml.

2.1.14 Confocal microscopy

Samples were viewed using a Zeiss photomicroscope III which was attached to the laser scan head (MRC-600, BioRad) fibre optically linked to an Argon ion 25mW laser. This illuminating light source emitted at the blue line (488nm) for excitation of FITC labeled antigens, a BHS filter set was inserted into the light path of channel 1. The contrast of images was adjusted via the photomultiplier gain to alter the black level. Image brightness was altered by adjusting the pinhole aperture, which was usually around two-thirds open. In all cases the contrast and brightness of the initial image was adjusted to use the maximum number of grey levels possible. Human blastocysts mounted in a glass microslides required a X40 long working distance lens (water immersion; Zeiss). Mouse blastocyst outgrowths were examined using a X60 PlanApo lens (oil immersion; Nikon). The computer operating system (COMOS) allowed control of the laser, microscope stage and image processing tools.

Single images and series were captured in the z-axis following Kalman averaging of typically 7 frames. For z-series of blastocysts, images were collected at 8-12 μ m z-steps (typically 15 optical sections) and images of trophoblast outgrowths were collected at 2 μ m z-steps (typically 5 optical sections). Images were stored on a rewritable optical disk (Panasonic) in the form of a BioRad pic file. Files were written to CD for permanent storage and manipulated using Confocal Assistant software.

2.2 MOLECULAR TECHNIQUES USED TO SUBCLONE $\beta 5$ cDNAs

2.2.1 Preparation of competent of *Escherichia coli* (E. coli) strains

To prepare competent MC1061/p3 cells, 50ml of Lauria Bertini (LB) medium (Appendix 2) containing kanamycin (40 μ g/ml) was inoculated with a stab of MC1061/p3 cells (Invitrogen). Cells were grown overnight at 37°C with in an orbital incubator (Gallenkamp) at 225rpm. Fresh LB medium (containing kanamycin) was inoculated with 400 μ l of cells and grown to mid-log phase (OD_{600nm} ~ 0.5). Cells were pelleted by centrifugation at 4000g for 10 min at 4°C. The pellet was resuspended in 10mls of filtered ice cold 0.1M CaCl₂, centrifuged again then resuspended in 2ml CaCl₂ solution and stored overnight at 4°C. Competent TOP10F' bacteria were purchased from Invitrogen.

2.2.2 Transformation of E. coli strains MC1061 and TOP10F'

100 μ l of competent bacteria (Table 2.3) per transformation were transferred to 15ml polypropanol tubes (Falcon) and 5 μ l of 0.5M β -mercaptoethanol was added. Cells were incubated on ice for 10 min and swirled gently every 2 minutes. Approximately 50ng (2.2.4) of purified plasmid DNA (2.2.10) was added to the bacteria and incubation on ice continued for a further 30 minutes. As a positive control for transformation 10ng of pUC18 plasmid DNA was added. Negative control bacteria had no plasmid DNA added. Bacteria were heat shocked at 42°C in a water bath for either 60 sec (MC1061/p3) or 30 sec (TOP10F') then returned to ice for 2 min. To each tube 900 μ l of pre-warmed SOC medium (Appendix 2) was added and bacteria were incubated at 37°C for 60 min with moderate agitation.

Bacteria were concentrated to a volume of 150 μ l after centrifugation at 4000g for 10 min. 100 μ l of transformed bacteria were spread onto LB agar plates (Appendix 2) containing 50 μ g/ml ampicillin (plus 10 μ g/ml tetracyclin for MC1061/p3) and incubated overnight at 37°C for selection of transformants. For blue/white colour selection of recombinant pCR2-EXTMp and pCR2-EXCYp transformants 44 μ l of X-Gal-isopropylthio- β -D-galactoside (IPTG) solution (Appendix 2) was spread on LB agar plates prior to adding bacteria.

Table 2.3 Key to plasmid terminology

| Plasmid name | Vector (supplier) | Insert | E. coli used for transformation |
|--|------------------------------|--|--|
| pcDNAneo1- β 5 (Scott Baldwin, Wistar Institute, Philadelphia) | pcDNAneo1 (Invitrogen) | Murine β 5 cDNA | MC1061/P3 (Invitrogen) |
| pCR2-EXCYp | pCR2 (Invitrogen) | Full length β 5 PCR product (EXCYp) | TOP10F' (Invitrogen) |
| pCR2-EXTMp | pCR2 | β 5 ectodomain PCR product (EXTMp) | TOP10F' |
| pAcSecG2T- EXCYbm | pAcSecG2T (Pharmingen) | Full length β 5 Bam H1 digest (EXCYbm) | TOP10F' |
| pAcSecG2T- EXTMbm | pAcSecG2T | β 5 ectodomain Bam H1 digest (EXTMbm) | TOP10F' |

2.2.3 Preparation of purified plasmid DNA

Single transformant colonies were picked from LB agar plates using a sterilized wire loop and used to inoculate 5ml of LB medium containing appropriate antibiotics (section 2.2.2). Cultures were grown overnight at 37°C with shaking (225rpm). Three millilitres of the culture was centrifuged at 15000g for 3 min and the supernatant was removed leaving a cell pellet from which plasmid DNA (Table 2.3) was purified. A Quiagen plasmid miniprep kit was used as described in the manufacturers instructions. Plasmid DNA was eluted with 75µl of either Tris EDTA buffer (TE; Appendix 2) or distilled water. The remaining 2ml of the cultures were stored at -80°C as glycerol stocks (Appendix 2).

2.2.4 Determination of DNA quantity and purity

DNA was quantified by measuring the absorbance of samples at 260nm using a UV spectrophotometer (GeneQuant DNA/RNA Calculator, Pharmacia). A concentration of 50µg/ml double stranded DNA gives an OD_{260nm} of ~1.

2.2.5 Restriction enzyme digestion

For isolation of DNA fragments for subcloning purposes the following reaction was used or scaled up as appropriate:

| | |
|-----------------------------|------|
| Plasmid DNA (500ng/µl) | 6µl |
| 10X enzyme buffer | 2µl |
| Restriction enzyme (10U/µl) | 2µl |
| Sterile water | 10µl |

The Bam HI, Hind III and Eco RI restriction enzymes (Boehringer Mannheim) were used, all are active at 37°C in the presence of Buffer B (Boehringer Mannheim). Digests were analyzed by agarose gel electrophoresis (2.2.6).

2.2.6 Agarose gel electrophoresis of DNA

DNA samples were analyzed by electrophoresis through gels of 1% (w/v) agarose (Gibco) in Tris acetate EDTA buffer (TAE; Appendix 2) containing 0.1 μ g/ml ethidium bromide. Samples were mixed with 6X loading buffer (Appendix 2) and loaded onto the gel along with appropriate molecular weight DNA standards. Electrophoresis was carried out at 80V in TAE buffer.

Gels were examined under ultra violet (UV) light to visualize fluorescing DNA and photographed (MP4 Land Camera, Polaroid) with high speed black and white film (Type 667, Polaroid).

2.2.7 Purification of DNA fragments by gel slice elution

After separation by agarose gel electrophoresis (2.2.6) the DNA fragment of interest was cut from the gel using a sterile scalpel. DNA was purified from a gel slice using the GeneClean II kit (Bio 101 Inc.), following manufacturers instructions. Briefly, DNA was eluted from the gel using a 6M sodium iodide solution and purified using the Glassmilk silica matrix which selectively binds single and double stranded DNA. The matrix was pelleted, washed and twice eluted with 5 μ l TE buffer or sterile water as appropriate.

2.2.8 Oligonucleotide primer design

Primers were designed to amplify both full length and the ectodomain of the mouse β 5 integrin subunit, with reference to a partial sequence of mouse β 5 cDNA kindly provided by Scott Baldwin (The Wistar Institute, Philadelphia). Three fragments of mouse β 5 sequence, termed MS β 5-1 (249bp), MS β 5-2 (237bp) and MS β 5-3 (374bp) have been aligned against human β 5 (GenBank accession code: M35011) and are shown in Figure 2.2.

[sequence encoding transmembrane domain is underlined; McLean *et al.*, 1990]

The primer sequences were as follows and were purchased from Gibco BRL:

Forward primer: EX (21-mer) 5'- **g↓ga tcc** atg ccg cgg gtg ccc -3'
 Reverse primer: CY (21-mer) 5'- **g↓ga tcc** tca gtc cac tga gcc -3'
 Reverse primer: TM (27-mer) 5'- **g↓ga tcc** tca ggc act tcc aca ttc tgg -3'

All primers included a Bam HI restriction enzyme site (bold type, ↓ shows cut site) at the 5' end which was used to clone β5 fragments into pAcSecG2T. The reverse primer TM also inserts a stop site (underlined) at the end of the β5 ectodomain. The forward primer EX in combination with the reverse primer CY was used to amplify full length β5 (2.2.9) including the β5 start and stop sites, producing a ~2.4Kb DNA fragment. When the forward primer EX and the reverse primer TM were used the ectodomain of β5 was amplified (2.2.9) producing a ~2.15Kb DNA fragment.

2.2.9 Polymerase chain reaction

After optimization of annealing temperature by trials between 60°C and 70°C the following 100μl reaction was used to amplify DNA fragments by polymerase chain reaction (PCR) from purified DNA preparations:

| | |
|-------------------------------|--|
| Template (1ng/μl) | 1-10μl |
| 10X PCR buffer | 10μl |
| dNTP's (25mM) | 1μl |
| Forward primer (10pM) | 6μl |
| Reverse primer (10pM) | 6μl |
| Taq polymerase | 1μl |
| DEPC treated H ₂ O | to make a final reaction volume of 100μl |

10X PCR buffer and Taq polymerase were obtained from Boehringer Mannheim. Samples were overlaid with a drop of mineral oil (Sigma) and incubated in a thermal cycler (PTC-100, M J Research Inc.) under the following conditions:

| | |
|--------------|-------------|
| Denaturation | 95°C, 1 min |
| Annealing | 67°C, 1 min |
| Elongation | 72°C, 1 min |

This was repeated for 35 cycles followed by a final elongation step at 72°C for 10 min. Samples were analyzed by agarose gel electrophoresis (2.2.6).

2.2.10 DNA Ligation

PCR generated DNA fragments (2.2.9) of full length (EXCYp) and the ectodomain (EXTMp) of $\beta 5$ were run on an agarose gel (2.2.6) and the appropriate bands were excised and eluted from the gel (2.2.7). For ligation of these fragments into the vector pCR2 a TA Cloning kit from Invitrogen was used. The ligation reaction was as follows:

| | |
|-----------------------------|---|
| PCR product (10ng/ μ l) | 1 μ l |
| pCR2 vector (25ng/ μ l) | 2 μ l |
| 10X ligation buffer | 1 μ l |
| T4 DNA ligase | 1 μ l |
| Sterile water | to make a final reaction volume of 10 μ l |

Samples were incubated overnight at 14°C and then used to transform competent TOP10F' E. coli (2.2.2).

DNA fragments generated by Bam HI digestion (2.2.5) of full length (EXCYbm) and the ectodomain (EXTMbm) of $\beta 5$ from the TA cloning vector pCR2 were separated on an agarose gel (2.2.6) and eluted from the gel slice (2.2.7). These fragments were subcloned into a baculovirus expression vector pAcSecG2T (20 μ g in TE buffer) which had been linearised by Bam HI digestion (2.2.5) and phosphatase treated (2.2.11). The ligation reaction of EXCYbm and EXTMbm into pAcSecG2T was as described above with the vector pAcSecG2T (25ng/ μ l) replacing pCR2 and inserts of 3 μ l of EXCYbm and EXTMbm (500ng/ μ l).

2.2.11 Phosphatase treatment of pAcSecG2T plasmid DNA

Linearised pAcSecG2T plasmid DNA (20 μ g in TE buffer) was extracted using phenol-chloroform (2.2.12) and precipitated with ethanol (2.2.13). The resulting pellet of linearised vector was resuspended in 85 μ l TE buffer and treated with calf intestinal phosphatase (CIP) to prevent self-ligation. The phosphatase reaction was as follows:

| | |
|---------------------------------|------------|
| Linear plasmid (50ng/ μ l) | 80 μ l |
| 10X CIP buffer (Sigma) | 10 μ l |
| CIP enzyme (1U/ μ l; Sigma) | 1 μ l |
| Sterile water | 69 μ l |

The mixture was incubated at 37°C for 45 min. To inactivate the CIP enzyme the following were added to the tube:

| | |
|-------------------------|------------|
| Sterile water | 75 μ l |
| STE buffer (Appendix 2) | 20 μ l |
| 10% (w/v) SDS | 10 μ l |

After incubation at 65°C for 20 min the DNA sample was cooled to room temperature, extracted twice with phenol-chloroform (2.2.12), precipitated using ethanol (2.2.13) and resuspended in 15 μ l sterile water. The linearised pAcSecG2T was then ligated to Bam HI digested β 5 DNA fragments (2.2.10).

2.2.12 Phenol-chloroform extraction

An equal volume of phenol chloroform saturated with isoamylalcohol (25:24:1; Sigma) was added to the DNA sample to be extracted. This was briefly vortexed, then centrifuged at 15000g for 5 min. The upper aqueous layer containing DNA was carefully removed and transferred to a new tube.

2.2.13 Ethanol precipitation

A 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of ice cold 100% ethanol were added to the DNA sample. Tubes were incubated overnight at -20°C to precipitate the DNA. Samples were centrifuged at 15000g for 20 minutes, supernatant was removed and the DNA pellet was washed with 200µl cold 70% ethanol and re-centrifuged. All traces of ethanol were removed, the DNA pellet was air dried for approximately 15 min. DNA was resuspended in 15µl of sterile water or TE buffer as appropriate.

2.2.14 Automated DNA sequencing

Partial sequencing of purified (2.2.3) pCR2-EXCYp and pCR2-EXTMp was carried out to confirm the presence of mouse $\beta 5$ integrin sequence. The ABI PRISM Dye Terminator Sequencing Kit (Perkin Elmer) was used as described in the manufacturers instructions in combination with M13 forward or reverse universal primers (Invitrogen). The sequences generated were analyzed using an ABI 373 DNA Sequencer (Wellcome Trust Unit, University of Manchester) and compiled using the Perkin-Elmer-Edit-View program (version 2.0).

2.3 PRODUCTION AND ANALYSIS OF RECOMBINANT PROTEINS

2.3.1 General handling and maintenance of the SF9 insect cell line

The SF9 clone of *Spodoptera frugiperda* cells (Pharmingen) was propagated at 27°C in Grace's medium supplemented with 3.3mg/ml lactalbumin hydrosylate and 3.3mg/ml yeastolate (Gibco BRL) containing 10% insect qualified FCS (Gibco BRL); termed 'complete growth medium'. Stock uninfected cells were cultured in 100mm plastic plates (Costar) and culture medium (10ml) was gently replaced three times a week. Cells were split 1:3 when a confluent monolayer had formed (weekly) by carefully dislodging attached cells using a stream of pre-warmed culture medium. The appropriate seeding densities for subconfluent monolayers in different sized cultures are shown in Table 2.4. When used, the antibiotics penicillin (100IU/ml; Gibco BRL), streptomycin (100µg/ml; Gibco BRL) and the antimycotic amphotericin B ('Fungizone', 2.5µg/ml; Gibco BRL) were added to culture medium. All manipulations were carried out under sterile conditions in a class 1 laminar air flow hood. All unwanted cells, conditioned medium, virus containing solutions and disposable culture equipment was decontaminated by treatment with chlorine bleach and/or incinerated.

2.3.1.1 Checking cell viability

Healthy SF9 cells are small, round and will readily attach to the culture dish, whereas baculovirus-infected cells are larger with swollen nuclei and detached from the culture dish. Cell viability was confirmed by adding a 0.3% stock solution of trypan blue (BDH) in PBS to an equal volume of a cell suspension. Cells were examined in a counting chamber (Hawksley), dead cells appear blue whereas healthy cells exclude the dye. In a healthy log-phase culture 97% of cells were alive.

2.3.1.2 Adapting cells to protein-free medium

When necessary, cells were grown in the absence of serum in the specially formulated culture medium SF-900II (proprietary formulation, Gibco BRL; termed 'SF growth medium'). Cells were adapted to serum-free medium by decreasing the ratio of complete growth medium to SF growth medium from 3:4 to 1:2 to 1:4 to SF growth medium alone, with each medium change.

Table 2.4 Culture of SF9 cells

| Culture dish or flask size | Seeding density | Culture volume | Technique used |
|-----------------------------------|------------------------|-----------------------|--|
| 100mm dish | 5×10^6 | 10ml | Routine cell culture Small scale protein production |
| 60mm dish | 2×10^6 | 4ml | Calcium phosphate co-precipitation co-transfection |
| 35mm dish | 1.5×10^6 | 3ml | Lipofectin co-transfection Plaque assay |
| 25cm ² flask | 1.5×10^6 | 4ml | Virus seed stock production |
| 75cm ² flask | 5×10^6 | 10ml | Virus working stock production |
| 500ml glass conical flask | 5×10^5 | 100ml | Initiation of suspension culture |
| 500ml glass conical flask | 2×10^5 | 100ml | Maintenance of suspension culture |
| 500ml glass conical flask | $1-2 \times 10^6$ | 100ml | Large scale protein production |

2.3.1.3 Adapting cells to suspension culture

Larger scale cultures were grown in 100ml culture volumes or in sterile 500ml glass conical flasks (Table 2.4). Flasks were initially seeded at a density of 5×10^5 cells/ml and grown to 3×10^6 cells/ml when they were reduced to 2×10^5 cells/ml. Cells were pelleted by gentle centrifugation at 600g for 10 minutes. Flasks were placed on an orbital shaker in a 27°C incubator maintained at a speed of approximately 80rpm.

2.3.1.4 Freezing and thawing cell stocks

Stocks of cells at a low passage number adapted to either complete or SF growth medium were stored under liquid nitrogen. Cells in fresh growth medium at 3×10^6 cells/ml were combined 1:1 with chilled freezing medium (80% growth medium containing 20% DMSO). The resulting cell suspension was split into 1ml aliquots, placed in cryovials (Costar) and frozen immediately in liquid nitrogen.

Cell were defrosted by removing a vial from liquid nitrogen and incubating in a 37°C water bath with agitation for approximately 1 min. When the ice had melted the vial was sterilized with 70% ethanol and the cells were decanted into a 100mm culture plate. A further 5ml of the appropriate growth medium was gently added to the dish and the cell suspension was evenly distributed over the base of the culture dish. Healthy cells were allowed to attach to the dish for 60 min and then the medium was replaced with 10ml of fresh growth medium, and cells were propagated at 27°C.

2.3.1.5 Counting cells

An improved Neubauer counting chamber was used to calculate cell density. A drop of cell suspension was placed over the grid ($1\text{mm}^2 \times 0.1\text{mm}$ depth), the coverslip was replaced and the number of cells was counted according to the manufacturers' instructions:

$$\text{Cells/ml} = \text{cell count} \times 10^4$$

$$\text{Total cell number} = \text{cell count} \times 10^4 \times \text{cell suspension volume}$$

For example,

$$150 \text{ cells} \times 10^4 = 1.5 \times 10^6 \text{ cells/ml}$$

$$150 \text{ cells} \times 10^4 \times 30\text{ml} = 4.5 \times 10^7 \text{ cells}$$

2.3.2 Co-transfection of SF9 cells to produce recombinant baculovirus

2.3.2.1 Calcium phosphate co-precipitation

In an attempt to co-transfect SF9 cells by calcium phosphate co-precipitation the BaculoGold Transfection Kit (Pharmingen) was used, as described in the manufacturers manual. Three 60mm dishes were seeded, providing plates for co-transfection, negative and positive controls. Meanwhile, 5 μ l of BaculoGold baculovirus DNA (0.1 μ g/ μ l; Pharmingen) was added to 50 μ l of purified plasmid DNA (either pAcSecG2T-EXCYbm or pAcSecG2T-EXTMbm at 0.1 μ g/ μ l). The DNA solution was vortexed and left at room temperature for 5 min, 1ml of Transfection buffer B (Pharmingen; containing 125mM HEPES (pH 7.1), 125mM CaCl₂, 140mM NaCl) was then added. The medium was removed from the co-transfection plate and replaced with 1ml of Transfection buffer A (Pharmingen; Grace's Medium with 10% FCS), ensuring all cells were covered. Transfection buffer B/DNA solution was added drop-wise to the plate with gentle rocking every four drops. This plate was incubated at 27°C for 4 hours after which time the co-transfection solution was removed and replaced with complete growth medium, this is termed day 1 of infection. The medium of the remaining two plates was replaced with 3ml fresh complete growth medium and to the positive control plate was added 50 μ l of wild-type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) supernatant (Pharmingen). All plates were then incubated at 27°C for a further 4 days. On day 5 of infection the conditioned medium was removed, spun at 15,000g for 10 min to remove cell debris and stored at 4°C in the dark.

2.3.2.2 Lipofectin mediated co-transfection

In order to increase the efficiency of co-transfection, a cationic lipid-mediated method utilizing Lipofectin (Gibco BRL) was used. For this three 35mm culture plates were seeded, providing plates for co-transfection, negative and positive controls as before. Meanwhile, 2 μ l of BaculoGold baculovirus DNA (0.1 μ g/ μ l; Pharmingen) was added to 10 μ l of SF growth medium. Approximately 1 μ g of purified plasmid DNA (0.2 μ g/ μ l) was added to another 10 μ l of SF growth medium. The two DNA solutions were mixed and made up to a final volume of 50 μ l by adding SF growth medium. The Lipofectin reagent (1mg/ml) was prepared by adding 17 μ l in a 1:3 ratio to SF growth medium (33 μ l). Finally, the DNA and Lipofectin solutions were combined in a 1:1 ratio and incubated at room temperature for 15 min. The medium was removed from the co-transfection plate and the cells were washed once with 2ml SF growth medium. To 900 μ l of SF growth medium, 100 μ l of the Lipofectin-DNA solution was added, this was mixed and added to the co-transfection plate. Following incubation at 27°C for 5 hours, 1 ml of complete growth medium was added to the co-transfection plate. This is termed day 1 of infection. The remaining two plates of cells were given 3ml of fresh complete growth medium and 25 μ l of wild-type AcMNPV virus supernatant (Pharmingen) was added to the positive control plate. The three plates were incubated at 27°C for a further 5 days. Conditioned medium was removed from the plates on day 6 of infection, spun and stored as above.

2.3.3 Determination of virus titre

2.3.3.1 Setting up the plaque assay

Eight 35mm dishes were seeded with cells; six of the dishes were used to determine recombinant virus titre, leaving two for the negative and positive plaque assay controls. The medium was removed from all plates, 150 μ l of complete growth medium was added to the negative control plate and 150 μ l of complete growth medium containing 1.5 μ l of wild-type AcMNPV virus supernatant (Pharmingen) was added to the positive control plate. The appropriate range of virus supernatant dilutions (10-fold, prepared in complete growth medium) was added in 150 μ l volumes to the remaining plates and incubated at room temperature for 60 min:

| | |
|------------------------------------|---|
| Co-transfection supernatant | 1:1 (undiluted) to 1:100,000 (10^{-5}) |
| Seed stock supernatant | 1:1,000 (10^{-3}) to 1:100,000,000 (10^{-8}) |
| Working stock supernatant | 1:10,000 (10^{-4}) to 1:1,000,000,000 (10^{-9}) |

To prepare the agarose overlay, 10ml of SF growth medium was placed in a sterile glass bottle, 0.18g of Agarplaque agarose (Pharmingen) was added and the solution was heated to 60°C until the agarose had dissolved (approximately 15 min). Once cooled to 40°C, 10ml of complete growth medium (containing 2X penicillin-streptomycin) was added to the agarose solution. Virus inoculum was removed from the plates and carefully replaced with 2ml of agarose overlay (cooled to 32°C to 37°C). Dishes were left with the lids removed while the agarose overlay dried (gels at 29°C). Once the agarose had set, 1ml of complete growth medium was added to each plate and the lids were replaced; this is termed plaque assay day 1. The plates were placed in a sandwich box lined with moist tissue paper and incubated at 27°C for a further six days.

2.3.3.2 Plaque identification and selection

For identification of plaques, plates are stained using neutral red stain (BDH). The stain is prepared by diluting 1ml of stock neutral red (0.5% w/v in PBS) to 1:20 in PBS and filter sterilized (0.45µm pore size; Gelman). To each plate 1.5ml of stain solution was added and plates were returned to the humid box and incubated at 27°C for 4 hours. After this time the stain solution was carefully removed, plates were inverted, placed in a sealed box and left at room temperature in the dark overnight to allow the stain to develop. Live cells will retain the stain and appear red whereas infected, lysed cells remain colourless.

At the appropriate dilution of virus, infected cells were seen as small colourless areas within the cell monolayer, where 10 to 30 discrete plaques can be identified. From this plate five plaques were picked using sterile 200µl pipette tips (Gibco). Each plaque pick was placed into 0.5ml of complete growth medium in a sterile bijou and kept dark by wrapping in foil. Virus particles were eluted from the agarose plug by rotating the tube overnight at 4°C.

2.3.3.3 Calculation of virus titre

Virus titre is calculated from the number of plaques seen on the plate of the appropriate virus dilution (where 10 to 30 discrete plaques are visible), as follows:

$$\text{Virus titre} = \text{plaque number} \times \text{virus dilution factor} \div \text{innoculum volume}$$

For example,

$$\begin{aligned}\text{Virus titre} &= 30 \text{ plaques} \times 100,000 \div 0.15\text{ml} \\ &= 2 \times 10^7 \text{ plaque forming units (PFU)/ml}\end{aligned}$$

2.3.3.4 End point dilution assay for assessment of virus titre

When attempting to titrate co-transfection virus supernatants which did not form visible plaques by plaque assay, an alternative and less accurate method based on an end point dilution assay (King & Posse; 1992) was adopted to determine the efficiency of virus in infecting smaller cell populations. A suspension of cells in complete growth medium at 2×10^5 cells/ml was prepared. Serial dilutions (1 in 10) of EXCY and EXTM co-transfection supernatants were made from neat to 10^{-4} and 100 μ l of each was added to 900 μ l of cell suspension. As controls, 100 μ l of wild type virus supernatant or 100 μ l complete growth medium were also added to cells. Each resulting 1ml cell suspension was added at 100 μ l volumes to the wells of a sterile 96-well plate (Costar), producing ten identical wells of each sample. The plate was examined daily to look for signs of virus infection and incubated for a total of 6 days at 27°C. On the sixth day of infection the proportion of each set of ten wells containing infected cells was determined. As this assay demonstrated a virtual absence of infectious virus in the co-transfection supernatants examined, the further calculations required for determination of virus titre were not used.

2.3.4 Amplification of virus titre from a plaque pick

2.3.4.1 Calculation of inoculum volume required

For amplification of viral stocks, cells are infected at a multiplicity of infection (MOI) of 0.1, that is 0.1 PFU/cell. The MOI is dependent on the titre of the virus stock and the number of cells to be infected, allowing the volume of inoculum required for amplification to be calculated as follows:

$$\text{Inoculum volume} = \text{MOI} \times \text{cell number} \div \text{virus titre}$$

For example,

$$\begin{aligned}\text{Inoculum volume} &= \text{MOI } 0.1 \times 5 \times 10^6 \text{ cells} \div 2 \times 10^7 \\ &= 0.025 \text{ ml}\end{aligned}$$

2.3.4.2 Amplification of virus titre

The medium was removed from an appropriately sized flask of cells (Table 2.5) and replaced with virus inoculum generated from a plaque pick (2.3.3) at an MOI of 0.1. The inoculum was made up to the required minimum volume (Table 2.5) with complete growth medium to ensure all cells were covered. Cells were incubated with virus at room temperature for 60 min, the inoculum was gently rocked over the monolayer every 15 min to prevent the cells from drying out. The inoculum was removed from the flask and replaced with complete growth medium (Table 2.5); this is termed day 1 of infection. Cells were incubated at 27°C until day 6 of infection, when the culture supernatant containing virus particles was removed and spun at 15,000g for 10 min to remove cell debris. The virus titre of the resulting supernatant was determined by plaque assay (2.3.3). A 1ml aliquot of each virus supernatant was stored at -80°C the remainder was stored at 4°C.

Table 2.5 Conditions for virus amplifications from plaque picks

| Flask size | Seeding density | Culture volume | Plaque pick source | Innoculum volume | Resulting virus stock |
|-------------------|------------------------|-----------------------|-----------------------------|-------------------------|------------------------------|
| 25cm ² | 1.5x10 ⁶ | 4ml | Co-transfection supernatant | 0.25ml | Seed stock virus |
| 75cm ² | 5x10 ⁶ | 10ml | Seed stock virus | 1ml | Working stock virus |

2.3.5 Examination of the SF9 genome for viral infection

2.3.5.1 Infection of cells with recombinant baculovirus

Three 60mm dishes were seeded with cells and used to prepare DNA from uninfected cells and cells infected with recombinant virus encoding full length $\beta 5$ (EXCY) or the ectodomain of $\beta 5$ (EXTM). The medium was removed from the cells and replaced with 0.25ml complete growth medium containing either recombinant virus at an MOI of 5, wild type virus or no virus. Cells were incubated with virus at room temperature for 1 hour and then the inoculum was replaced with 4ml fresh complete growth medium; this is termed day 1 of infection.

2.3.5.2 Extraction and analysis of genomic DNA

On day 6 of infection the cells were scraped into the medium and pelleted by centrifugation at 1,000g for 5 min. The supernatant was removed and the cell pellet was washed with 500 μ l of PBS. The cells were again pelleted and then resuspended in 250 μ l of TE buffer. To this, 250 μ l of SF9 DNA lysis buffer (50mM Tris HCl pH 7.5, 5% (v/v) β -mercaptoethanol, 0.4% (w/v) SDS, 10mM EDTA pH 8), 12.5 μ l proteinase K (10mg/ml at 37°C) and 2.5 μ l of ribonuclease A (Boeringer Mannheim) was added. The solution was incubated at 37°C for 30 min then extracted twice with phenol-chloroform (see 2.2.12) and ethanol precipitated (2.2.13). The resulting DNA pellet was resuspended in 100 μ l of TE buffer. After quantification of the DNA preparation (2.2.4) PCR was carried out to confirm the presence of the EXCY and EXTM transcripts within the infected SF9 cell genome, as described in section 2.2.9.

2.3.6 Small scale extraction of SF9 cell proteins using SDS

Ten 100mm dishes were seeded with cells, two served as uninfected controls while eight were infected with recombinant baculovirus, four with EXCY and four with EXTM. Briefly, the culture medium was removed and replaced with virus supernatant (MOI 10) or control, made up to a volume of 1ml with fresh growth medium. Cells were incubated with virus for 60 min after which time the inoculum was replaced with 10ml fresh growth medium; termed day 1 of infection.

Dishes were incubated at 27°C and removed on days 2, 3, 4 and 5 of infection. Uninfected dishes were removed on days 2 and 5 only. Once removed from the incubator cells were scraped into the medium using a cell scraper (Costar) and pelleted by centrifugation at 500g for 5 min at 4°C; from this stage cells and medium were kept on ice unless otherwise stated. The medium was collected and further centrifuged at 15,000g for 10 min at 4°C. The clarified medium was then aliquoted and either stored at -20°C or concentrated approximately 25-fold prior to storage using centricon-10 protein concentrators (Millipore) as described in the manufacturers instructions. Meanwhile, the cell pellet was washed in 500µl ice cold PBS, pelleted again then resuspended in 80µl TE buffer. To the resulting cell slurry 20µl of 'SDS lysis buffer' (50mM Tris buffer pH 6.9, 25% glycerol (v/v), 10% SDS (w/v)), containing protease inhibitors (1µM pepstatin A, 1mM EDTA and 50mM PMSF) was added. DNA released following nuclear lysis was broken down by passing the sample through a 26 gauge needle (Beckton Dickenson) attached to a 1ml syringe (Terumo) ten times. Samples were then centrifuged at 15,000g for 15 min at 10°C, the supernatant was aliquoted and stored at -20°C. Medium and cell lysate samples were analysed by polyacrylamide gel electrophoresis (2.3.12).

2.3.7 Extraction of SF9 cell proteins without SDS

2.3.7.1 Small scale extraction of SF9 cell proteins without SDS

Four 100mm dishes were seeded with cells, two were infected with recombinant baculovirus EXTM and the remaining dishes were uninfected controls. As described previously (2.3.6), the culture medium was removed and replaced with virus supernatant or control in a volume of 1ml complete growth medium. After 60 min the inoculum was replaced with 10ml fresh growth medium. Cells were cultured until day 3 of infection.

Once removed from the incubator cells were scraped into the medium using a cell scraper (Costar) and pelleted by centrifugation at 500g for 5 min at 4°C. The cell pellets of one infected and one uninfected dish were mixed with 2ml ice cold 'Triton X-100 lysis buffer A' (TX-A; 50mM Tris buffer (pH 8) containing 100mM NaCl and 1% (v/v) Triton X-100; courtesy of N. Smithers, Glaxo Wellcome) and

incubated on ice for 15 min. The lysates were then pulse sonicated (Ultrasonic Heat Systems, Life Science Laboratories Ltd, Luton). Following lysate centrifugation at 14000g for 15 min at 4°C, the supernatant was retained and the pellets were discarded. Three millilitres of 'Triton X-100 lysis buffer B' (TX-B; 50mM Tris (pH 8) containing 150mM NaCl and 1% (v/v) Triton X-100; Nolan & Morgan, 1995) was added to the cell pellets of the remaining two dishes. These lysates were incubated on ice for 20 min and then centrifuged at 14000g for 15 min at 4°C. The supernatant (termed 'cytoplasmic fraction') was removed and the remaining pellets were then further lysed in 2ml TX-B containing 1% (w/v) SDS (TX-B+SDS; termed 'nuclear fraction') for 20 min on ice. The nuclear fraction was then centrifuged as before. The various lysates of infected and uninfected cells generated under alternative lysis conditions were analysed by polyacrylamide gel electrophoresis.

2.3.7.2 Large scale extraction of SF9 cell proteins using detergent

Cells grown as 100ml suspension cultures were propagated to a density of 2×10^6 cells/ml prior to infection. Twelve flasks of cells were individually concentrated to 2×10^7 cells/ml in fresh serum-free growth medium for infection with recombinant baculovirus at an MOI of approximately 5. After incubation at 27°C for 1 hour cells were diluted down to 2×10^6 cell/ml with additional fresh serum-free growth medium and incubated until 72 hours post-infection.

For collection of infected cells for lysis, cultures were split between twenty-four 50ml tubes (Falcon) and centrifuged in groups of four at 700g for 15 min at 4°C. Cell pellets (derived from two 100ml flasks) were washed in 5ml of ice cold sterile PBS, repelleted and resuspended in PBS to a volume of approximately 2.4ml. Six hundred microlitre aliquots of cell slurry were spread evenly over the surface of 100mm culture dishes on ice. Cells were lysed at a 10:3 ratio of original culture volume to lysis buffer (50mM Tris pH 8, 150mM NaCl, 1% (v/v) Triton-X100; section 2.3.7.1, TX-B) containing protease inhibitors (2.3.6), where 15ml of lysis buffer was added to each 600µl of cell slurry. The cell slurry was incubated in lysis buffer for 15 min and occasionally swirled to ensure all cells were lysed. Cell lysates were collected, pooled and centrifuged at 5,500g for 10 min at 4°C to

pellet unlysed cell debris and the resulting 360ml of supernatant was split equally between ten 50ml tubes for purification of GST-tagged fusion proteins (2.3.8).

2.3.8 Purification of fusion proteins using a glutathione affinity column

Glutathione Sepharose 4B (GS4B; Pharmacia Biotech) was used to purify β 5 integrin fusion proteins by affinity to the glutathione-S-transferase (GST) tag, using the Pharmacia Biotech GST purification module. The kit is designed for purification of GST from bacterial sonicates therefore the methods described below were adapted from the manufacturers instructions.

2.3.8.1 Preparation of glutathione Sepharose 4B

GS4B is provided as a 75% slurry in ethanol and is prepared by draining off the ethanol and washing three times in 10 bed volumes of PBS, producing a 50% GS4B slurry. A GS4B bed volume is equivalent to three-quarters of a volume of 75% slurry or half a volume of 50% slurry. For purification, a bed volume of 50 μ l GS4B per 100ml SF9 culture or 30ml cell lysate was used.

2.3.8.2 Binding of recombinant protein to glutathione column

For binding of fusion proteins present in cell lysates an appropriate bed volume of GS4B was added to the lysate at a 1:600 ratio of equilibrated beads to lysate and left overnight with gentle agitation at 4°C.

To purify from cell conditioned medium an appropriate bed volume of GS4B (1:2000 beads to original culture volume) was packed into a disposable column and the medium was twice passed through the column at approximately 10ml/hour using a controlled delivery pump (Pharmacia Biotech) at 4°C.

2.3.8.3 Elution of GST from glutathione Sepharose 4B

For elution of GST-tagged proteins from GS4B beads in the cell lysates, the beads were first sedimented by centrifugation at 500g for 5 min at 4°C. The supernatant was carefully discarded, the bead slurries were combined and split equally before packing into two disposable columns prior to elution. For elution of proteins from conditioned medium the beads were already packed into the

column for the GST binding step.

The column was washed and drained three times with 10 bed volumes of ice cold sterile PBS. Fusion protein was then eluted with 10mM glutathione elution buffer (10mM glutathione in 50mM Tris buffer pH 8) by incubation of the GS4B with 1.5 bed volumes of elution buffer for 10 min at 4°C followed by draining of the columns into a sterile eppendorf, this step was repeated twice. This was followed by a third elution step where the column was incubated in 10mM glutathione elution buffer for 25 min before draining. Presence of eluted fusion protein was determined by a Bradford protein assay, also using a GST-activity assay (2.3.8.4) and by polyacrylamide gel electrophoresis followed by silver staining and Western blotting (2.3.12).

2.3.8.4 GST detection assay

A GST detection assay (Pharmacia Biotech) utilizing the GST substrate 1-chloro-2,4-dinitrobenzene (CNDB) was used to assess levels of fusion protein in samples by measuring GST enzyme activity through its ability to conjugate CNDB with glutathione, resulting in a product with strong molar absorbtion at 340nm. The assay reagent is prepared by mixing 100µl of 10X reaction buffer (1M phosphate buffer, pH 6.5), 10µl of CNDB solution (100mM CNDB in ethanol) and 10µl of glutathione solution (100mM glutathione) with 880µl of distilled water. Five hundred microlitres of assay reagent was transferred to each of two UV-transparent cuvettes. To the 'sample cuvette' 10 or 20µl of the sample to be assayed was added. The 'blank cuvette' was set to an absorbance of zero at 340nm and the sample cuvette was then measured, this was repeated a further four times at one minute intervals by first blanking the spectrophotometer. The results from the CNDB assay were then used to calculate the change in absorbance per minute per ml of sample to determine relative GST levels:

$$\Delta A_{340\text{nm}}/\text{min/ml} = [A_{340\text{nm}}(t_5) - A_{340\text{nm}}(t_1)] \div [(t_5 - t_1) \times \text{ml sample added}]$$

Where: $A_{340\text{nm}}(t_n)$ = absorbance at 340nm at n minutes

For example,

$$\Delta A_{340\text{nm}}/\text{min/ml} = [0.705 - 0.185] \div [4 \text{ min} \times 0.02\text{ml}] = 6.5$$

2.3.9 Determination of protein concentration

2.3.9.1 Lowry protein assay

In order to quantify total protein in cell lysates and medium samples the Pierce BCA protein assay (Pierce), based on the Lowry method (Lowry *et al.*, 1951) was used. Standard BSA protein samples ranging from 1mg/ml to 0.015mg/ml and serially diluted test samples were loaded at 20 μ l into the well of a 96-well plate (Costar). To each well 200 μ l of BCA protein reagent (diluted 1:50 in distilled water) was added and plates were incubated at 37°C for 30 minutes. Plates were read at 570nm on a microtitre plate reader (Dynatech) and protein concentration was determined by comparing test samples to a BSA standard curve.

2.3.9.2 Bradford protein assay

To quantify protein in solutions containing glutathione the Biorad protein assay based on the Bradford method (Bradford, 1976) was used at two levels of sensitivity. To detect 0.2 to 1mg/ml of protein, duplicate standard BSA samples of 0.2, 0.4, 0.6, 0.8 and 1mg/ml were prepared, for detection of 100 to 500 μ g of protein duplicate BSA standards of 100, 200, 300, 400 and 500 μ g/ml were used. Test samples remained undiluted. The Biorad reagent was prepared by dilution 1:5 in water. The assay consisted of either 3 μ l of sample/standard and 150 μ l of Biorad reagent (for detection of 0.2 to 1mg/ml protein) or 10 μ l of sample/standard and 90 μ l of Biorad reagent (for 100 to 500 μ g/ml protein), loaded into a 96-well plate (Costar). The plate was immediately read at 630nm and test sample protein concentrations were determined by comparison with the appropriate BSA standard curve constructed by first averaging the duplicate BSA absorbance readings.

2.3.10 Polyacrylamide gel electrophoresis

Protein-containing samples were separated under reducing conditions according to the method of Laemmli (1970). Separating (7.5%) and stacking (4%) gels (0.75mm thickness) were prepared using stock solutions (Table 2.6), where the APS and TEMED were added just prior to pouring the gels. The stacking gel was allowed to polymerize for at least 30 min before overlaying with stacking gel. Protein samples to be analysed were mixed with 10% (v/v) β -mercaptoethanol, 10% (v/v) bromophenol blue (in ethanol) and made up to a volume of 30 μ l with electrode running buffer. Samples were then boiled for 10 minutes and centrifuged at 15000g for 15 min prior to being loaded onto the gel. Broad range standard molecular weight markers (BioRad; Table 2.7) were also loaded, allowing prediction of sample protein sizes. Electrophoresis was performed using the BioRad minigel system with electrophoresis running buffer (Appendix 3), at a constant current of 15 to 20mA for approximately 90 min. Molecular weights of sample proteins were extrapolated from a log curve of standard molecular weight plotted against distance traveled through gel.

Table 2.6 Preparation of polyacrylamide gels

| Component | 7.5% Separating gel | 4% Stacking gel |
|----------------------------------|----------------------------|------------------------|
| Distilled water | 4.85ml | 6.1ml |
| 1.5 M Tris HCl pH 8.8. | 2.5ml ^e | - |
| 0.5 M Tris HCl pH 6.8 | - | 2.5ml ^f |
| 10% (w/v) SDS stock ^a | 100μl | 100μl |
| 30% Acrylamide/Bis ^b | 2.5ml | 1.3ml |
| 10% APS ^c | 50μl | 50μl |
| TEMED ^d | 5μl | 10μl |

^a Sodium dodecyl sulphate (BDH)

^b Contains 2.6% N, N'-methylene-bisacrylamide cross linker (BioRad)

^c Ammonium persulphate (BioRad)

^d N, N, N', N'-tetramethylethylenediamine (BioRad)

^e Resulting in a 375mM Tris buffer (pH 8.8)

^f Resulting in a 125mM Tris buffer (pH 6.8)

Table 2.7 Protein standard markers

| Protein standard | Molecular weight / kDa* | |
|---------------------------|-------------------------|-------|
| | Lot 1 | Lot 2 |
| myosin | 209 | 200 |
| β -galactosidase | 137 | 117 |
| bovine serum albumin | 84 | 75 |
| carbonic anhydrase | 44 | 42.9 |
| soybean trypsin inhibitor | 32 | 31.8 |

*As multicoloured dye-linked markers (BioRad) were used the standard molecular weight of proteins was variable between lots.

2.3.10.1 Coomassie staining of gels

To evaluate protein separation and loading (typically 25µg or 50µg per lane) gels were stained with 0.1% (w/v) Coomassie Blue (Sigma) dissolved in 45% (v/v) distilled water, 45% (v/v) methanol (BDH) and 10% (v/v) glacial acetic acid (BDH) for 1 hour. Gels were then placed in destain (50% (v/v) methanol, 40% (v/v) distilled water and 10% (v/v) glacial acetic acid) until the background staining had been removed (approximately 4 hours). The destained gels were rehydrated for 1 hour in several changes of distilled water and then dried.

2.3.10.2 Silver staining of gels

A more sensitive method for silver staining of proteins (25µg or less per lane) was used with scarce samples. Once run the gel was placed in fixative (60% (v/v) distilled water, 26% (v/v) ethanol, 14% (v/v) formaldehyde) overnight at 4°C. After washing for 30 min in 5 changes of distilled water the gel was reduced in 5µg/ml dithiothreitol (BioRad) for 15 min. The gel was then stained in 0.1% (w/v) silver nitrate for 15 min in the dark. After the gel was rapidly washed twice with distilled water, then rinsed twice in developer (0.05% (v/v) formaldehyde and 3% (w/v) anhydrous sodium carbonate (BDH) in distilled water) it was incubated in a further change of developer over a light box until protein bands were visible (5 - 10 min). The reaction was stopped by replacing the developer with a 2.3M solution of monohydrous citric acid (BDH) and incubated for 15 min. Finally the gel was washed in distilled water for 30 min before drying.

2.3.11 Western blotting

2.3.11.1 Protein transfer

Gels to be analysed by Western blotting were first transferred to nitrocellulose membrane (BioRad) using a the Transblot wet blotting system (BioRad) and transfer buffer containing 25mM Tris HCl (pH 6.8), 192mM glycine, 0.01% (w/v) SDS and 20% methanol. Prior to transfer the gel and nitrocellulose membrane were equilibrated in chilled transfer buffer for 30 min at 4°C. Transfer of proteins to the membrane was for 60 min at 100V. Following transfer the membrane was incubated in blocking buffer (PBS containing 10% (w/v) fat-free milk powder (Tesco) and 0.3% (v/v) Tween-20 (Sigma)) overnight at 4°C.

2.3.11.2 Antibody binding

The membrane was incubated in 5 to 20ml of primary antibody (Table 2.1) diluted in blocking buffer for 90 min with gentle agitation. The membrane was rinsed twice with wash buffer (PBS containing 0.3% (v/v) Tween-20) and then incubated twice for 10 min in 25ml wash buffer with gentle agitation. The membrane was incubated in 20ml of secondary antibody (Table 2.2) for 90 min with gentle agitation and washed as before. Finally the membrane was washed twice for 10 min with distilled water prior to detection.

2.3.11.3 Chemiluminescent detection

Proteins were detected by enhanced chemiluminescence (ECL, Amersham). The ECL reagent was prepared by mixing the two components 1:1, this was gently placed onto the protein-side of the membrane (5ml) and left for exactly 1 min. The membrane was drained, wrapped in Saranwrap (Dow Chemicals) and placed protein side up in a developing cassette (Sigma). In the dark the blot was exposed to autoradiographic film (Hyperfilm-ECL; Amersham) as appropriate (30 sec to 60 min). The film was transferred to developer (Kodak LX24) for 2 min, rinsed, placed in fixative (Kodak FX40) for 1 min, rinsed again and air dried.

CHAPTER 3

Expression and regulation of α_v integrins in endometrium

3.1 INTRODUCTION

Previous studies have identified many integrins in human endometrium. Most are constitutively expressed throughout the menstrual cycle, while some (notably $\alpha v\beta 3$) show a regulated expression pattern. $\alpha v\beta 3$ expression appears during the secretory phase on human endometrial glands and is potentially a marker of uterine receptivity (section 1.7.6). As part of my study the expression pattern of a closely related integrin, $\alpha v\beta 5$, was investigated in human endometrium. αv family integrins (αv , $\beta 3$ and $\beta 5$ subunits) were also examined in the mouse uterus. Endometrial tissue was taken at each stage of the non-pregnant estrus cycle, and from days 1 to 6 of pregnancy covering the pre-, peri- and post-implantation stages. In addition endometrium was examined from mice that had undergone ovariectomy followed by steroid hormone replacement, to determine whether these integrins are regulated by oestrogen and/or progesterone. The results described in this chapter (and additional work) were recently published (Aplin *et al.*, 1996).

3.2 RESULTS

3.2.1 Preliminary investigations

An immunocytochemical investigation of integrin subunit expression was carried out on cryopreserved human and mouse endometrial tissue (sections 2.1.1, 2.1.2). Prior to undertaking the complete study, various parameters in the immunocytochemistry protocol (section 2.1.3) were optimised. The required antibody dilutions were determined (Tables 2.1/2.2). Secondly, the efficacy of alternative primary antibody detection methods were examined. It was found that a biotin-conjugated secondary antibody followed by FITC-conjugated streptavidin provided amplification of fluorescence signal over that generated using a FITC-conjugated secondary antibody. For this study, the three-step staining protocol was adopted (section 2.1.3).

Integrin subunit expression on the luminal and glandular epithelium and stromal cells of the endometrium were examined. The apical, basal and lateral epithelial cell surfaces were studied and staining intensity was classified as high '++',

moderate '+' or weak '(+)'. Occasionally discrete patches of high intensity staining '++P' were seen within an area of weak or moderate staining. Where present the staining of blood vessels 'BV' is also described.

3.2.2 Human endometrium

Due to the difficulty in obtaining fresh human endometrial tissue the following results were obtained from a limited number of samples (3 patients). Human endometrium was obtained by dilatation and curettage for routine investigation of infertility (2 patients) and in association with laproscopic sterilisation (1 patient); therefore endometrial samples could be considered as abnormal. Nevertheless samples could be histologically dated with respect to the stage in the menstrual cycle, by the criteria of Noyes et al (1950). $\beta 5$ subunit expression throughout the menstrual cycle in samples of normal human endometrium (from wax-embedded archive material) was subsequently investigated by Ljiljana Vicovac in our laboratory (see Aplin *et al.*, 1996).

3.2.2.1 $\alpha v\beta 5$ integrin expression in human endometrium and decidua

The integrin $\alpha v\beta 5$ is present in human endometrium around the period of receptivity and during the first trimester of pregnancy (Figure 3.1; Table 3.1). Cryopreserved mid-secretory phase human endometrium expressed $\beta 5$ highly on glandular epithelium and moderately within the stroma (Figure 3.1b). Expression of $\beta 5$ appeared equivalent at the apical, basal and lateral surfaces of the glandular epithelial cells. No examples of luminal epithelium were preserved in this tissue. A similar pattern of expression was seen for αv (Figure 3.1a). A heterodimer-specific antibody to $\alpha v\beta 5$ detected moderate levels of this integrin on glands and low levels within the stroma. In first trimester endometrial decidua, αv and $\beta 5$ were retained by the glandular epithelium (Figures 3.1 c/d) and stroma, at a similar level and distribution. In decidual tissue, high expression of both subunits was seen in blood vessels and the peri-vascular stroma (Figures 3.1e/f). Some sections had pre-immune rabbit serum or PBS instead of primary antibody, these served as negative controls. Representative examples of negatively stained human endometrium and decidua are shown in Appendix 6.

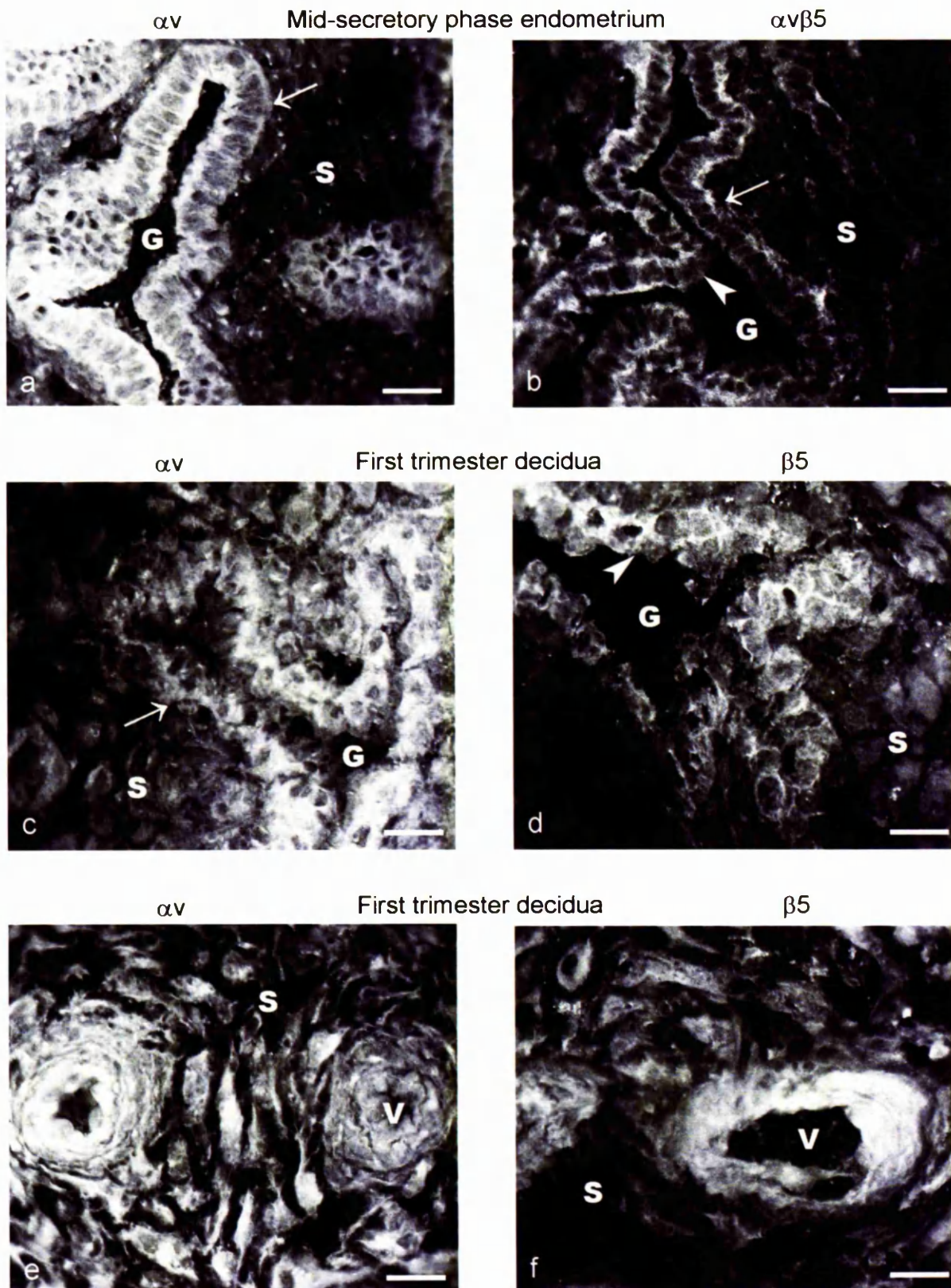


Figure 3.1 Immunolocalisation of αv and $\beta 5$ integrins in human endometrium
 Integrin subunits αv and $\beta 5$ are expressed by epithelium and stroma around the time implantation and during early pregnancy, see 3.2.2.1. Scale bar = 20 μm

G, gland; **S**, stroma; **V**, blood vessel; \blacktriangleright apical surface; \longrightarrow basal surface

Table 3.1 $\alpha_v\beta_5$ integrin expression in human endometrium and decidua

| Antibody specificity (name) | Mid-secretory phase endometrium | | First trimester decidua | |
|-----------------------------------|---------------------------------|--------|----------------------------|----------|
| | Epithelium | Stroma | Epithelium | Stroma |
| α_v (MAB1978) | ++ | + | ++ | + (BV++) |
| β_5 (AB1926) | ++ | + | + / ++ | + (BV++) |
| $\alpha_v\beta_5$ (MAB1961) | + | (+) | + | (+) |

[++, high staining; +, moderate staining; (+), weak staining; (++)BV blood vessels]

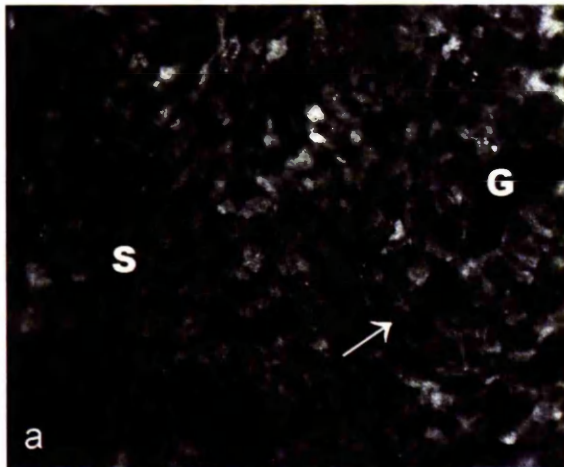
3.2.3 Mouse endometrium

In each experiment, tissue sections incubated in pre-immune rabbit serum in the place of primary antibody were also included. These proved negative for immunofluorescence in each case. A representative example of negative control endometrium is shown in Figure 3.2a. This image is of proestrus stage endometrium, the lack of staining demonstrated is equivalent to that observed in mouse endometrium at any other stage of the estrus cycle, during early pregnancy or in ovariectomised animals.

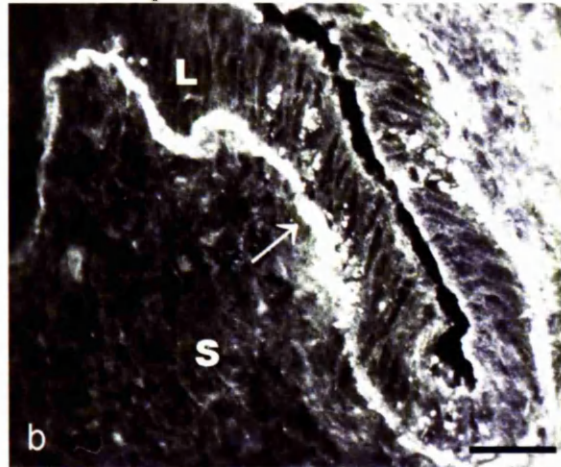
3.2.3.1 $\beta 5$ subunit expression

Expression of $\beta 5$ (AB1926) was seen in the epithelium and stroma at all stages (Figure 3.2; Table 3.2). Stromal expression of $\beta 5$ was moderate in all samples. (e.g. Figure 3.2b). Moderate levels of $\beta 5$ appear at the apical surface of luminal (e.g. Figure 3.2c) and glandular epithelium (Figure 3.2e). The basal surface of glands were generally weakly stained for $\beta 5$, but the basal surface of luminal epithelium was always highly immunopositive for $\beta 5$ (Figure 3.2d). Little variation in the expression-or distribution of $\beta 5$ was seen between samples of non-pregnant or pregnant endometrium at different stages. Furthermore, $\beta 5$ appears to be expressed independently of steroidal regulation (Table 3.2).

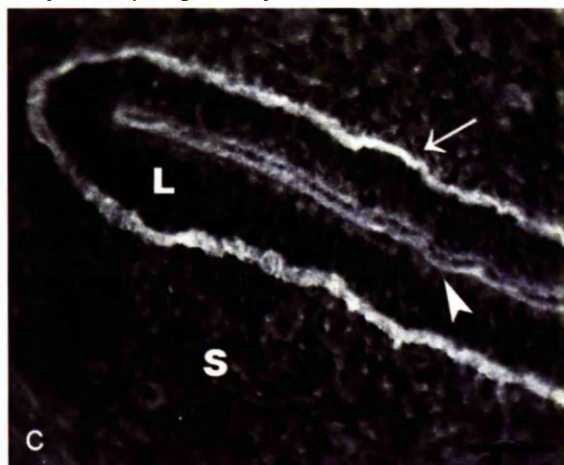
Proestrus stage - negative control



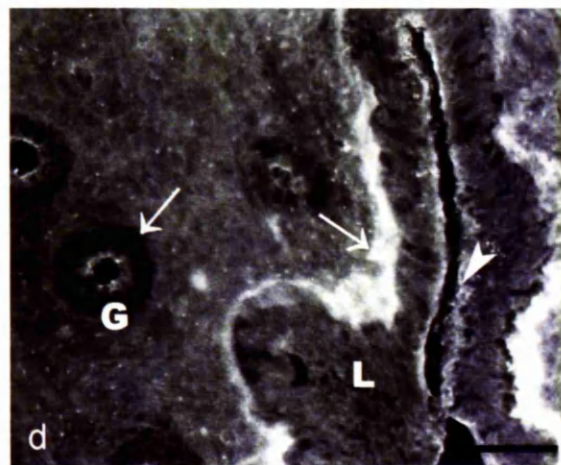
Estrus stage



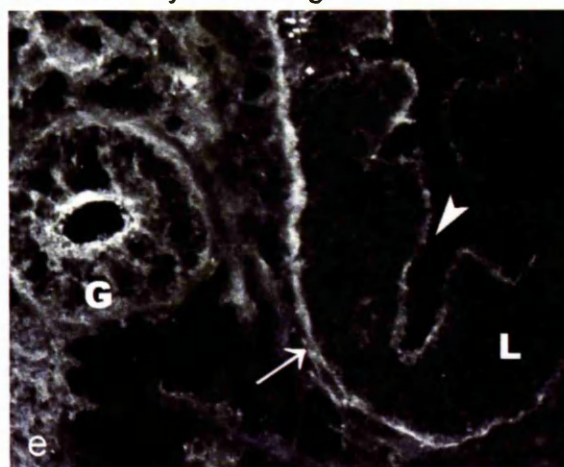
Day 5 of pregnancy



Ovariectomy + vehicle



Ovariectomy + oestrogen



Ovariectomy + progesterone

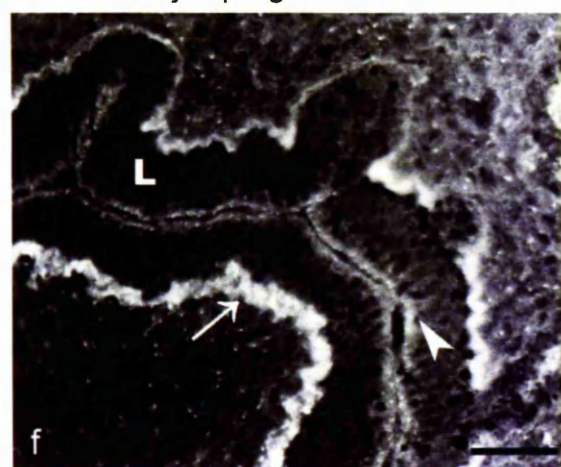


Figure 3.2 Immunolocalisation of $\beta 5$ integrins in mouse endometrium

Integrin $\beta 5$ is expressed by the apical and basal epithelium, and stroma during the estrus cycle and early pregnancy. It is not regulated by steroids, see 3.2.3.1. Scale bar = 20 μ m

L, lumen; **G**, gland; **S**, stroma; \blacktriangleright apical surface; \longrightarrow basal surface

Table 3.2 $\beta 5$ integrin subunit expression in mouse endometrium

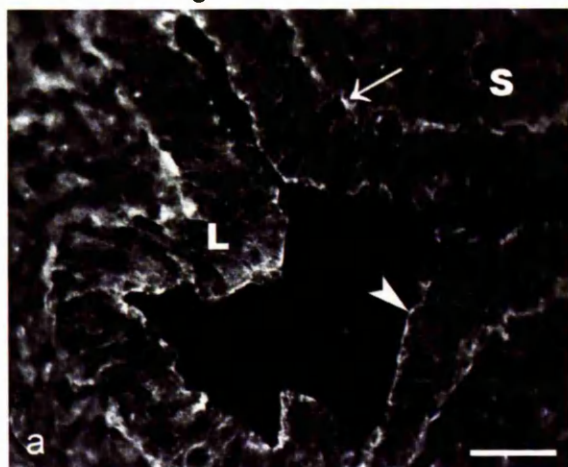
| | Apical luminal epithelium | Basal luminal epithelium | Apical glandular epithelium | Basal glandular epithelium | Lateral epithelium | Stroma |
|---|---------------------------------|--------------------------------|-----------------------------------|----------------------------------|-----------------------|--------|
| Estrus cycle | | | | | | |
| Proestrus | + | ++ | + | + | (+) | + |
| Estrus | + | ++ | + | (+) | + | + |
| Metestrus | + | ++ | + | + | + | + |
| Diestrus | + | ++ | + | (+) | (+) | + |
| Early pregnancy | | | | | | |
| Day 1 | + | ++ | + | (+) | ND | + |
| Day 2 | + | ++ | + | (+) | (+) | + |
| Day 3 | + | ++ | + | (+) | (+) | + |
| Day 4 | + | ++ | + | (+) | (+) | + |
| Day 5 | + | ++ | + | (+) | ND | + |
| Day 6 | + | ++ | + | (+) | ND | + |
| Ovariectomy with steroid hormone replacement | | | | | | |
| No hormone | + | ++ | + | (+) | (+) | + |
| E ¹ | + | ++ | + | (+) | (+) | + |
| E ¹ & P | + | ++ | + | (+) | (+) | + |
| E ¹ & P & E ² | + | ++ | + | (+) | + | + |

[++, high staining; +, moderate staining; (+), weak staining; ND, not determined;
E¹, oestrogen priming; E², nidatory oestrogen]

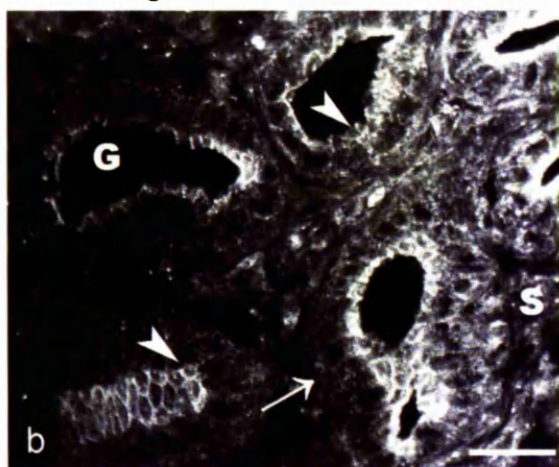
3.2.3.2 $\beta 3$ subunit expression

$\beta 3$ (AB1932) expression was detected in all endometrial samples, in both the epithelium and stroma (Figure 3.3; Table 3.3). Stromal cells had consistently moderate levels of $\beta 3$ (e.g. Figure 3.3b). The apical surface of the epithelium stained more intensely for $\beta 3$ than the basal surface at most stages (e.g. Figure 3.3d). In ovariectomised animals, injection of progesterone (following oestrogen priming) may have corresponded to slightly increased $\beta 3$ at the apical surface, although this was not clearly demonstrated on single micrographs (Figure 3.3f). This was independent of nidatory oestrogen. Similar slight increases in $\beta 3$ were seen from the estrus to metestrus stage of the non-pregnant endometrial cycle (Figure 3.3c) and from day 4 of pregnancy (Figure 3.3d). These possible alterations of $\beta 3$ levels were seen in the luminal and glandular epithelium of non-pregnant or ovariectomised animals. Elevation of $\beta 3$ appears mainly restricted to the luminal epithelium during pregnancy (Figure 3.3d; Table 3.3).

Proestrus stage



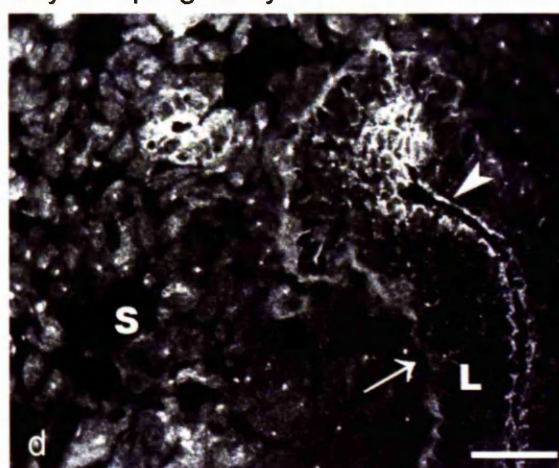
Estrus stage



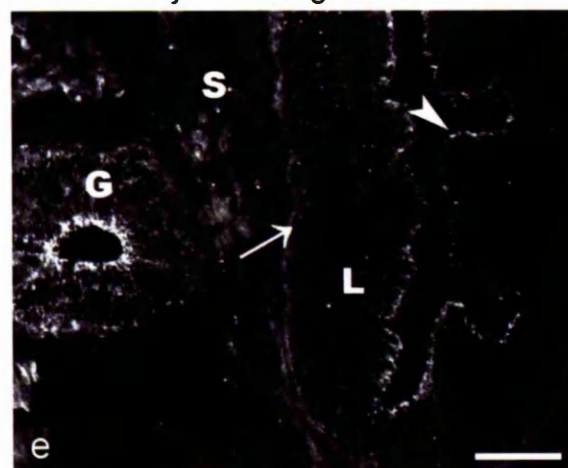
Day 3 of pregnancy



Day 4 of pregnancy



Ovariectomy + oestrogen



Ovariectomy + progesterone



Figure 3.3 Immunolocalisation of $\beta 3$ integrins in mouse endometrium

Integrin $\beta 3$ is expressed by the apical epithelium, and stroma during the estrus cycle and early pregnancy. It appears unregulated by steroids, see 3.2.3.2. Scale bar = 20 μ m

L, lumen; **G**, gland; **S**, stroma; \blacktriangleright apical surface; \longrightarrow basal surface

Table 3.3 $\beta 3$ integrin subunit expression in mouse endometrium

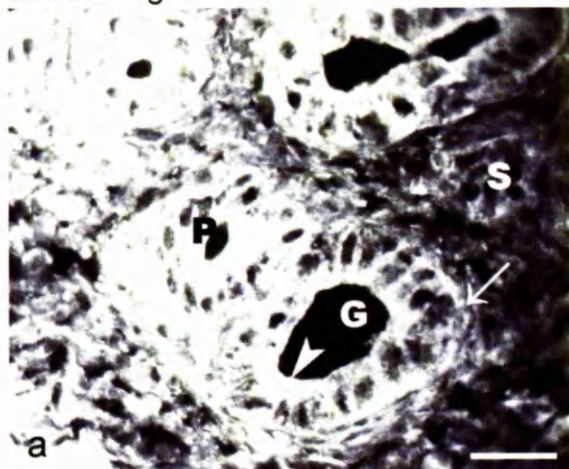
| | Apical luminal epithelium | Basal luminal epithelium | Apical glandular epithelium | Basal glandular epithelium | Lateral epithelium | Stroma |
|---|---------------------------------|--------------------------------|-----------------------------------|----------------------------------|-----------------------|--------|
| Estrus cycle | | | | | | |
| Proestrus | + | + | + | + | + | + |
| Estrus | +/++ | + | +/++ | (+) | (+) | + |
| Metestrus | ++ | + | ++ | + | (+) | (+) |
| Diestrus | + | + | + | (+) | (+) | + |
| Early pregnancy | | | | | | |
| Day 1 | + | + | + | (+) | ND | + |
| Day 2 | + | (+) | + | (+) | (+) | + |
| Day 3 | + | (+) | + | (+) | (+) | + |
| Day 4 | +/++ | + | +/++ | (+) | (+) | (+) |
| Day 5 | ++ | (+) | + | (+) | ND | + |
| Day 6 | ++ | + | + | (+) | ND | + |
| Ovariectomy with steroid hormone replacement | | | | | | |
| No hormone | + | + | + | (+) | (+) | (+) |
| E ¹ | + | + | + | + | + | (+) |
| E ¹ & P | ++ | + | ++ | (+) | + | + |
| E ¹ & P & E ² | ++ | + | ++ | (+) | + | + |

[++, high staining; +, moderate staining; (+), weak staining; ND, not determined;
E¹, oestrogen priming; E², nidatory oestrogen]

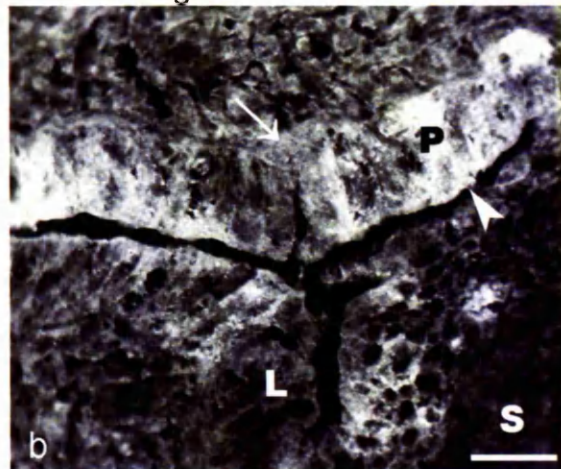
3.2.3.3 αv subunit expression

Weak to moderate staining for αv (AB1930) was detected in all endometrial samples, in both the epithelium and stroma (Figure 3.4; Table 3.4). The basal surface of the epithelium generally stained less than the apical surface, at both the luminal (e.g. Figure 3.4d) and glandular epithelium (e.g. Figure 3.4f). During the estrus cycle and on days 2 and 3 of pregnancy, occasional patches of very fluorescent αv -positive cells were seen (e.g. Figure 3.4b). These were mostly restricted to the epithelium and often covered the whole cell. Other than these unusually bright patches, no significant change in staining intensity or distribution was seen at different stages of the estrus cycle or during early pregnancy. In ovariectomised animals, replacement of oestrogen alone, oestrogen and progesterone (with or without subsequent nidatory oestrogen), did not significantly alter αv subunit levels relative to untreated controls.

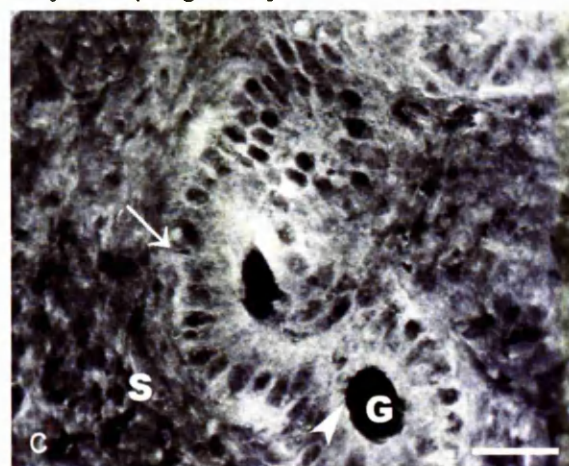
Estrus stage



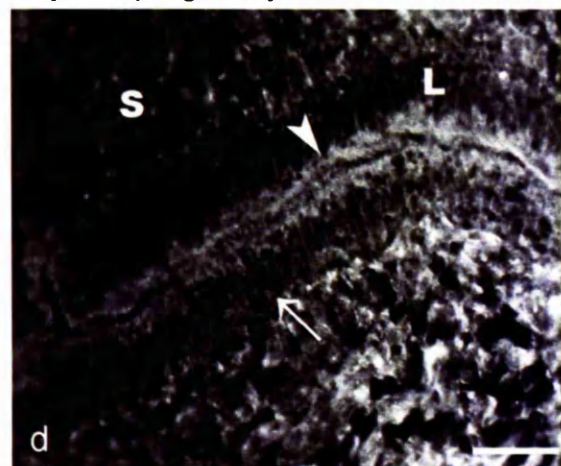
Diestrus stage



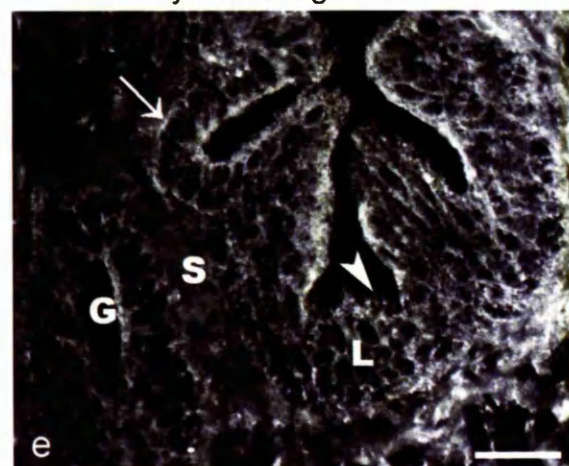
Day 1 of pregnancy



Day 5 of pregnancy



Ovariectomy + oestrogen



Ovariectomy + progesterone

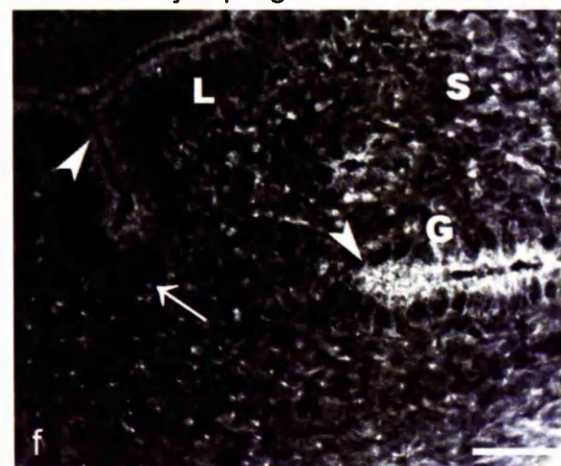


Figure 3.4 Immunolocalisation of αv integrins in mouse endometrium

Integrin αv is expressed by the epithelium and stroma during the estrus cycle and early pregnancy. It appears not to be steroidally regulated, see 3.2.3.3. Scale bar = 20 μ m

L, lumen; **G**, gland; **S**, stroma; **P**, bright patch; \blacktriangleright apical surface; \longrightarrow basal surface

Table 3.4 αv integrin subunit expression in mouse endometrium

| | Apical luminal epithelium | Basal luminal epithelium | Apical glandular epithelium | Basal glandular epithelium | Lateral epithelium | Stroma |
|---|---------------------------------|--------------------------------|-----------------------------------|----------------------------------|-----------------------|---------|
| Estrus cycle | | | | | | |
| Proestrus | + | + | + | (+) | + | + |
| Estrus | + | + | + (++P) | (+) (++P) | + (++P) | + |
| Metestrus | + (++P) | + | + (++P) | + | + (++P) | + |
| Diestrus | + (++P) | (+) (++P) | + (++P) | (+) (++P) | (+) | + |
| Early pregnancy | | | | | | |
| Day 1 | + | (+) | + | (+) | ND | + |
| Day 2 | + (++P) | (+) (++P) | + | (+) | (+)(++P) | + |
| Day 3 | + | (+) | + | (+) (++P) | (+) (++P) | + (++P) |
| Day 4 | + | (+) | + | (+) | (+) | + |
| Day 5 | + | (+) | + | (+) | + | + |
| Day 6 | + | (+) | + | (+) | + | + |
| Ovariectomy with steroid hormone replacement | | | | | | |
| No hormone | + | (+) | + | (+) | + | + |
| E ¹ | + | + | + | (+) | + | + |
| E ¹ & P | + | (+) | + | (+) | + | + |
| E ¹ & P & E ² | + | (+) | + | (+) | (+) | + |

[++, high staining; +, moderate staining; (+), weak staining; (++P) patches of differentially stained cells; ND, not determined; E¹, oestrogen priming; E², nidatory oestrogen]

3.3 DISCUSSION

The $\beta 5$ integrin subunit and its only known partner αv have been identified in human endometrium during the mid-secretory phase. This short study was limited by the availability of fresh human endometrial tissue for cryopreservation and subsequent immunofluorescent analysis. The expression of the αv integrin subunit did not differ from that reported previously (Lessey *et al.*, 1992, 1994a). Following the observation of $\beta 5$ (and αv) at high levels in glands and moderate levels in the stroma of mid-secretory phase endometrium, expression of $\beta 5$ during the menstrual cycle was studied further in our laboratory (Aplin *et al.*, 1996). For this, wax embedded archive human endometrial material and an alternative immunodetection method were used. This work confirmed the expression pattern of $\beta 5$ in mid-secretory phase endometrium. The luminal epithelium was preserved in the wax-embedded samples, and luminal expression of $\beta 5$ paralleled that seen in the glands. $\beta 5$ showed a predominantly apical localisation at the luminal and glandular epithelium (Aplin *et al.*, 1996). No significant variation in $\beta 5$ subunit expression was detected at any stage during the menstrual cycle, suggesting a lack of steroidal regulation. This is consistent with the continued expression of $\beta 5$ (and αv) on glandular epithelium of first trimester decidua, which differentiates in response to hormonal changes that occur with pregnancy.

Using a dimer-specific monoclonal antibody to human $\alpha v\beta 5$, the presence of this integrin in the endometrium was confirmed. However, staining was less intense than when antibodies to either subunit alone were used. Another study using an $\alpha v\beta 5$ dimer-specific antibody failed to detect $\alpha v\beta 5$ in human endometrium (Lessey *et al.*; 1996). This paper also described little expression of $\alpha v\beta 6$, an integrin which has been clearly demonstrated in other studies (Breuss *et al.*, 1995). These apparent discrepancies may be due to the differences in the epitopes detected by the different antibodies or by their binding kinetics. The $\beta 5$ specific polyclonal antibody is expected bind multiple epitopes on the integrin subunit and therefore is more likely to detect the antigen sensitively, although it is raised to a relatively short peptide. Also it is possible that it contains high affinity

antibodies, which produce high sensitivity. The monoclonal anti- $\alpha v\beta 5$ (MAB1961) has function blocking activity in vitro and probably recognises an epitope in the ligand-binding region (Bergman *et al.*, 1995; Wayner *et al.*, 1991). When an integrin is bound to its ligand, or is present in fixed, frozen tissue sections, the ligand-binding region may be difficult to access, which may in part explain the reduced staining seen using this antibody. Also, for an antibody to be effective in immunofluorescence it should not dissociate rapidly from antigen so that it is not simply washed away during post-incubation steps. Such binding kinetics are not necessarily required for good function blocking activity where the antibody concentration is maintained in excess in the cellular environment. Therefore an antibody with recognised function blocking activity may not be efficient at revealing the same integrin in tissue sections.

Differentiative changes of uterine spiral arteries commence during the secretory phase of the menstrual cycle and continue if pregnancy is achieved (Tang *et al.*, 1994). Increased $\beta 5$ and αv expression was a characteristic of peri-vascular stroma and blood vessels undergoing decidual transformation. Elevated peri-vascular $\alpha v\beta 5$ correlates with increased COL IV, LM-1 and HSPG (Aplin *et al.*, 1988), although none of these ECM components are known ligands for $\alpha v\beta 5$. FN is also a major component of the stromal and peri-vascular environment (Aplin *et al.*, 1988) and is the most likely ligand for $\alpha v\beta 5$ in this location. VN, an alternative ligand for $\alpha v\beta 5$, is only weakly expressed in the human endometrium. OST is also a ligand for $\alpha v\beta 5$ (along with $\alpha v\beta 1$ and $\alpha v\beta 3$) and is upregulated in secretory phase endometrium in the epithelium (Brown *et al.*, 1992). In humans TSP is greatly upregulated in the stroma and vascular basement membrane as decidualisation of the endometrium begins (Iruela-Arispe *et al.*, 1996), and is expressed during pregnancy in the mouse (Corless *et al.*, 1992). TSP has so far only been identified as a ligand for $\alpha v\beta 3$, but $\alpha v\beta 5$ shares certain ligands with $\alpha v\beta 3$. In the future, TSP (and other ECM components) may be demonstrated as alternative ligands for $\alpha v\beta 5$.

The lack of significant hormonal control of β_5 and α_v in human endometrium suggested a follow-up investigation where experimental steroidal requirements could be imposed in the mouse. The unchanging expression pattern seen during the estrus cycle or early pregnancy suggested that they were independent of hormonal control. This was confirmed using endometrial tissue taken from ovariectomised animals after different steroid replacement regimes. An unusual feature of α_v expression in the mouse endometrium was occasional intensely stained patches of epithelium seen in some samples. This pattern was not seen with either β_5 or β_3 and showed no hormonal regulation. Although the expression of other α_v partners (β_1 , β_6 or β_8) was not examined, the unlikely distribution of the staining over the whole cell cytoplasm suggests that it is an artifact. Staining with an alternative antibody reactive with mouse α_v would help resolve this point.

A striking difference between mouse and human expression of β_5 is the highly immunopositive basal surface of the luminal epithelium seen in mouse endometrium. This pattern of β_5 localisation is not seen in mouse glandular epithelium, or any human endometrial epithelium. α_v is only weakly present at the basal luminal epithelium, and the pattern of staining seen for α_v and β_5 , excluding that seen at the basal luminal epithelium, are virtually identical. One possible explanation is that β_5 may pair with an alternative and as yet unidentified α subunit, at least in the mouse, that may be involved in anchoring the luminal epithelium to its basement membrane. The endometrial basement membrane consists primarily of LM and COL IV (Blankenship & Given, 1995), themselves not known to be $\alpha_v\beta_5$ ligands. α_6 is a potential additional partner for β_5 that has prominent localisation at the basal epithelial surface in human endometrium (Lessey *et al.* 1992). However, α_6 usually associates with β_4 or β_1 . The observation that high basal expression of β_5 in the endometrium is only seen at the luminal epithelium suggests that it may be of particular importance in implantation. Potentially it may help maintain the integrity of the epithelial barrier. By examining β_5 expression in mouse early implantation sites it may be possible to determine whether this integrin is locally modulated by the implanting embryo.

Several studies have examined the expression of αv family integrins (including $\alpha v\beta 5$) in different tissues (Agrez *et al.*, 1996; Nikkari *et al.*, 1995; Milner & French-Constant, 1994). As most have used dimer-specific antibodies, there has been little opportunity for alternative binding partners for $\beta 5$ to be discovered. Several groups have immunoprecipitated $\alpha v\beta 5$ from cell lysates using an anti- $\beta 5$ antibody, without demonstrating additional α subunits (Delannet *et al.*, 1994; Adams & Watt, 1991). In the most revealing study, six novel anti-human $\beta 5$ monoclonal antibodies were used for quantitative analysis of the respective levels of αv and $\beta 5$ at the cell surface (Pasqualini *et al.*, 1993). Equivalent levels of both subunit were identified on several human cell lines. However, a lung carcinoma cell line (A549) had up to 3-fold more $\beta 5$ than αv at the cell surface. This was demonstrated with each of the anti- $\beta 5$ antibodies. On these cells, an anti- $\alpha v\beta 5$ antibody showed the dimer to be present at levels equivalent to that seen for αv , indicating an alternative $\beta 5$ -partner might be present. But, when A549 cells were immunoprecipitated with anti- αv , anti- $\alpha v\beta 5$ or any of the anti- $\beta 5$ antibodies, similar protein complexes were revealed. Furthermore when all αv protein was immunodepleted with anti- αv , no detectable anti- $\beta 5$ reactivity remained. Therefore expression of $\beta 5$ by A549 cells greatly exceeded that of αv for unknown reasons (Pasqualini *et al.*, 1993). To further investigate this hypothesis $\beta 5$ expression will need to be examined further, for example by immunoprecipitation of uterine tissue or primary epithelial cell cultures.

In contrast to αv or $\beta 5$, the expression of $\beta 3$ in the mouse uterus does show subtle changes which may reflect hormonal regulation. Slight elevation of $\beta 3$ is seen at the apical surface of the endometrial epithelium. In the non-pregnant mouse this begins during the estrus stage and is most obvious at metestrus (see Figure 1.1). In early pregnancy in the mouse, progesterone levels steadily rise from 48 hours after mating (equivalent to day 3 of pregnancy; see section 1.2.2). Increased $\beta 3$ is seen from day 4 of pregnancy and maintained until at least day 6, suggesting the possibility of progesterone regulation. This hormonal control was confirmed in endometrium from ovariectomised animals. Here, injection of progesterone (following oestrogen priming) correlated with increased $\beta 3$ at the

apical surface, irrespective of subsequent nidatory oestrogen. In baboon, $\beta 3$ is expressed by glandular epithelial cells only in pregnancy, or in ovariectomised animals given prolonged replacement of oestrogen with progesterone (Fazleabas *et al.*, 1997). Thus providing additional evidence for steroidal regulation of this integrin. The apparent hormonally modulated expression of $\beta 3$ at the endometrial epithelium in the mouse and baboon, in part supports previous observations made in human endometrium, although here the mechanisms seem more complex. $\beta 3$ has been described as a marker of endometrial receptivity as it appears on epithelial cells during the secretory phase of the menstrual cycle, when progesterone is the dominant hormone (Lessey *et al.*, 1992, 1994a, 1996). However $\beta 3$ appears abruptly on day 20, a pattern which would require more precise regulation than simply elevated progesterone. In vitro studies using an human endometrial carcinoma cell line show that $\beta 3$ expression is downregulated by oestrogen (Somkuti *et al.*, 1997). This does not seem to be the case in mouse endometrium because in ovariectomised animals following progesterone replacement, subsequent injection of nidatory oestrogen does not appear to reverse the rise in $\beta 3$. It has been suggested that endometrial expression of integrins in humans is not directly dependent on oestrogen or progesterone action (Sillem *et al.*, 1997). Various studies have therefore examined the role of local growth modulators in regulating human endometrial $\beta 3$. In vitro experiments using endometrial carcinoma cell lines have suggested that in humans $\alpha v \beta 3$ is upregulated by EGF (Somkuti *et al.*, 1997). Also, co-cultures of isolated human endometrial epithelial cells and human pre-implantation stage embryos indicate that healthy blastocysts increase the expression of $\beta 3$ by the endometrial cells, and that this may be mediated by the IL-1 system (Simon *et al.*, 1997).

In summary, $\beta 3$, $\beta 5$ and αv are present at the apical membrane of luminal epithelium in the mouse uterus, the surface to which an implanting embryo will initially attach. The ligand binding activity of these endometrial integrins has yet to be directly demonstrated. $\beta 3$ at the endometrial epithelial surface appears to

be regulated by progesterone (in the mouse) and therefore it could be important in endometrial receptivity. This study contributed to the first detailed examination of $\beta 5$ expression by endometrial tissue (Aplin *et al.*, 1996) and provided clear evidence for a lack of steroidal control of $\beta 5$ (and αv) in the mouse uterus. It also demonstrated the possible existence of an alternative binding partner for $\beta 5$. The basal location of $\beta 5$ at the luminal epithelium in the mouse endometrium suggests it may play a role in early trophoblast invasion perhaps by controlling epithelial displacement following embryo attachment, a feature of rodent implantation.

CHAPTER 4

Expression of αv integrins in early embryos

4.1 INTRODUCTION

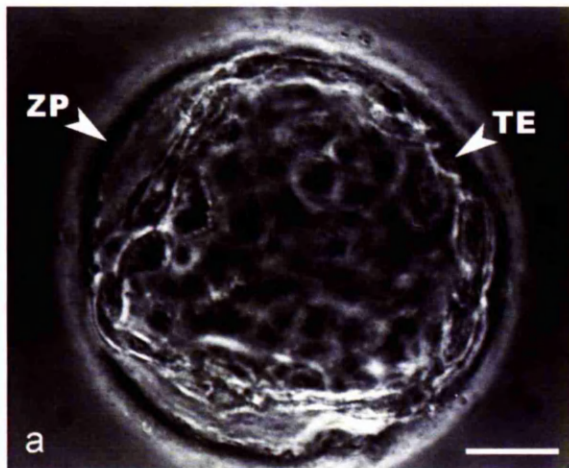
Various integrins have been identified on mouse early embryos. Some are constitutively expressed (e.g. $\alpha 5 \beta 1$, $\alpha v \beta 3$). Others appear developmentally regulated (section 1.7.6). Integrins have also been examined in human pre-implantation stage embryos (section 1.7.6). Through the course of my work I was fortunate to receive a selection of human blastocysts with normal morphology. This rare material, surplus to requirements in IVF treatment regimes, was used to study the expression of αv family integrins in human peri-implantation stage embryos. To allow species comparison to be made, mouse blastocysts were also stained for αv family integrin subunits. Having identified a potential role for these integrins in implantation, their expression was studied further in an in vitro model of early implantation. Here, mouse blastocysts were allowed to attach to a surface coated with serum proteins, resulting in an outgrowth of trophoblast cells. By examining the distribution of the αv , $\beta 1$, $\beta 3$ and $\beta 5$ integrin subunits, their potential function in early trophoblast invasion could be assessed.

4.2 RESULTS

4.2.1 Human blastocysts

Human embryos generated from IVF treatment regimes were donated for experimental use. The blastocysts were fixed, following non-invasive in vitro metabolic studies by researchers at York University (section 2.1.4). The zona pellucida was not removed from human blastocysts prior to staining, so as to keep manipulation to a minimum (Figure 4.1a). Approximately 25 blastocysts (Table 4.1) were stained (section 2.1.5). Stained embryos were examined by confocal microscopy (section 2.1.14). Multiple images were collected through the z-plane and then reconstructed to produce the z-series projection, as seen in Figure 4.1b-f.

Integrin αv - phase contrast



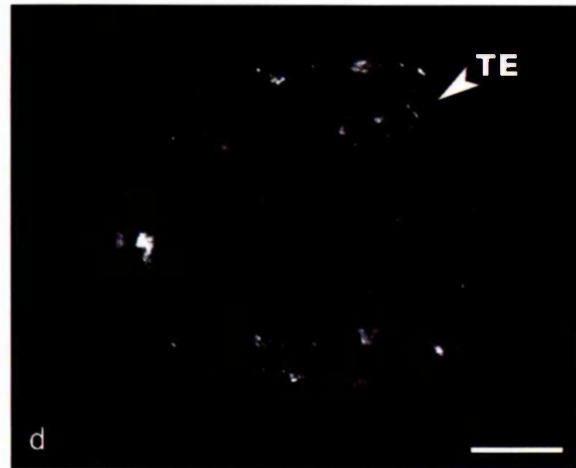
Negative control



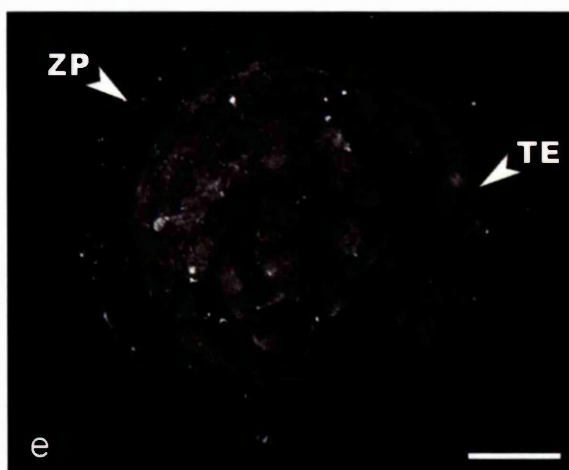
Integrin αv



Integrin $\beta 1$



Integrin $\beta 3$



Integrin $\beta 5$

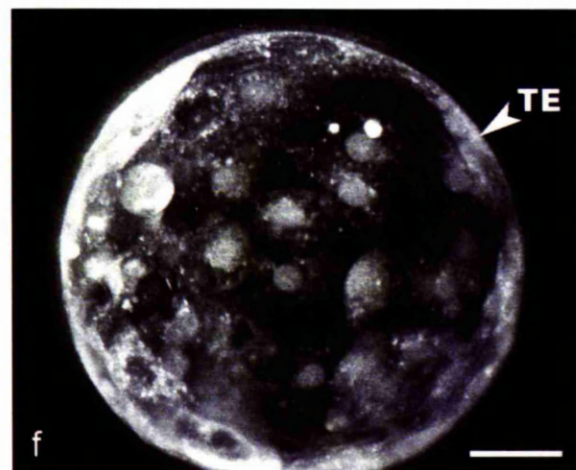


Figure 4.1 Immunolocalisation of αv integrins in human blastocysts

Human embryos generated in IVF cycles were cultured in vitro to the blastocyst stage

Confocal images collected in the Z-plane were compiled and projected flat (b-f)

The blastocyst shown by phase contrast (a) is also seen in (c), see 4.2.1

ZP, zona pellucida; **TE**, trophectoderm epithelium; scale bar = 50 μm

Table 4.1 Integrin subunit expression by human blastocysts

| Integrin subunit (antibody) | αv (MAB1978) | $\beta 1$ (MCA1188) | $\beta 3$ (AB1932) ^{CY} | $\beta 5$ (AB1926) ^{CY} | $\alpha v\beta 5$ (MAB1961) |
|---------------------------------------|-------------------------|------------------------|-------------------------------------|-------------------------------------|--------------------------------|
| Number stained | 4 + | 2 + | 2 + | 3 + | 1 (+) |
| and intensity | 2 (+) | 1 (+) | 2 (+) | 1 – | |

[+, moderate; (+), weak; –, negative; ^{CY}, antibody to integrin cytoplasmic domain]

4.2.1.1 αv family integrin expression by human blastocysts

All integrins studied (αv , $\beta 1$, $\beta 3$, $\beta 5$, $\alpha v\beta 5$ dimer) were found to be expressed by human peri-implantation stage embryos. Staining intensity ranged from weak to moderate (Figure 4.1; Table 4.1). Overall the staining pattern seen using antibodies to integrin extracellular domains (Figures 4.1c/d) was different to that seen using antibodies to the cytoplasmic tail. In the latter case it was necessary to permeabilise embryos prior to staining, resulting in cytoplasmic staining throughout the embryo (Figures 4.1e/f). Anti-extracellular domain antibodies produced a more punctate staining pattern, restricted to the external trophoctoderm surface. Several embryos were incubated in pre-immune rabbit serum (Figure 4.1b) or PBS in place of primary antibody, these showed no significant immunofluorescence in either case.

4.2.2 Pre- and peri-implantation stage mouse embryos

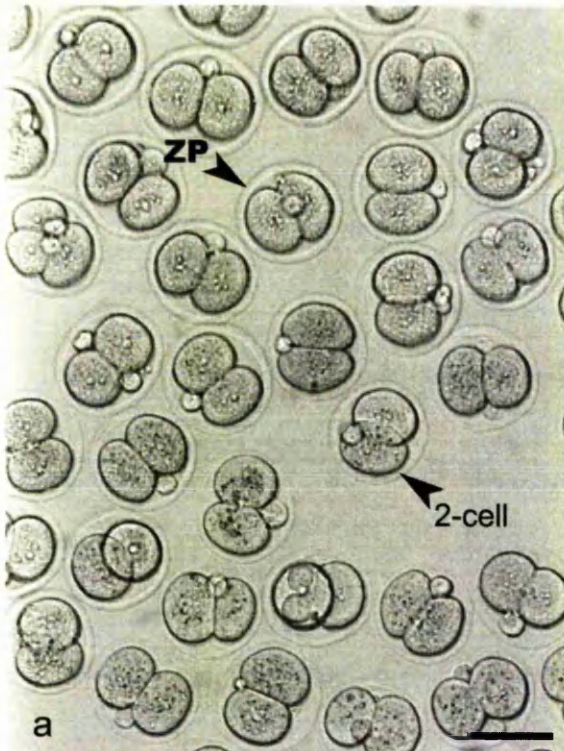
Mouse embryos were cultured in vitro from the two-cell stage to blastocysts (section 2.1.6). Embryos were taken from culture at the blastocyst (or occasionally morula) stage for immunofluorescence. As a greater supply of mouse embryos was available, they were routinely stained in groups of 30 for each integrin subunit and this was repeated on at least four occasions (section 2.1.7). Mouse embryos were examined by phase contrast, light and epifluorescence microscopy. Images were collected in the form of black and white photographs, and in some cases digital images were also captured.

4.2.2.1 Development of mouse embryos in vitro

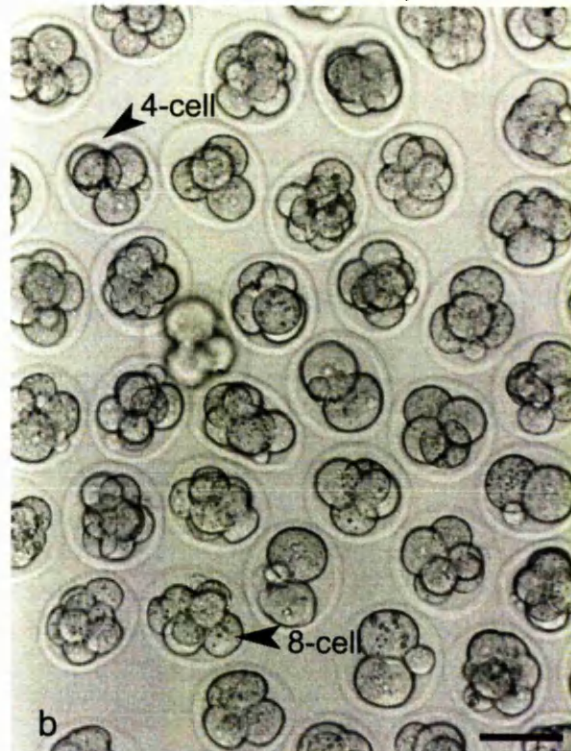
Embryos were collected from mouse oviducts on day 2 of pregnancy and maintained in vitro in simple medium. At this stage (termed 'day 2 of development') the majority of fertilised oocytes were at the 2-cell stage (Figure 4.2a). By late day 2 to early day 3, viable embryos had divided to become 4-cells embryos. Later on day 3 embryos divided again becoming 8-cell embryos (Figure 4.2b). On day 4, embryos at several developmental stages could be seen. These ranged from compacted 8-cells to early blastocysts, but the majority appeared to be at the morula stage (Figure 4.2c). During day 5 advanced embryos became blastocysts, but most were still undergoing the morula-to-blastocyst transition.

By day 6 all healthy embryos had reached the blastocyst stage. They had become fully expanded and were at various stages in hatching from the zona pellucida (Figure 4.2d).

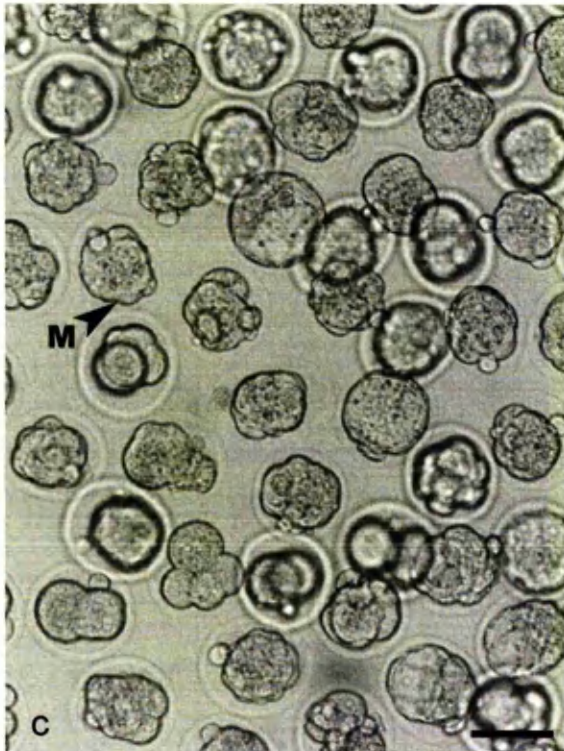
Day 2 2-cell embryos



Day 3 4-cell to 8-cell embryos



Day 4 Compacted 8-cell to morula



Day 6 Expanded, hatched blastocysts

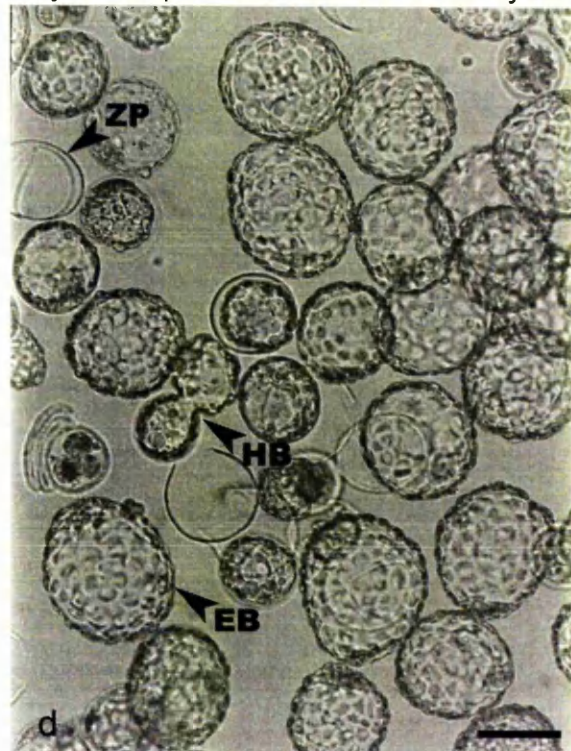


Figure 4.2 Development of mouse embryos in vitro.

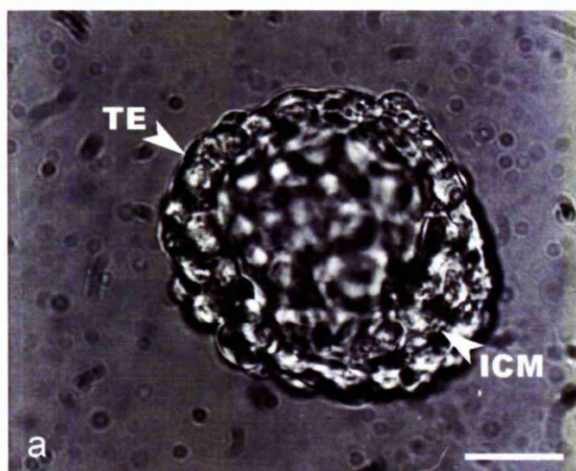
Embryos collected at the 2-cell stage and maintained in vitro to the blastocyst stage
Shown by phase contrast microscopy, see 4.2.2.1. Scale bar = 100 μ m

ZP, zona pellucida; **M**, morula; **HB**, hatching blastocyst; **EB**, expanded blastocyst

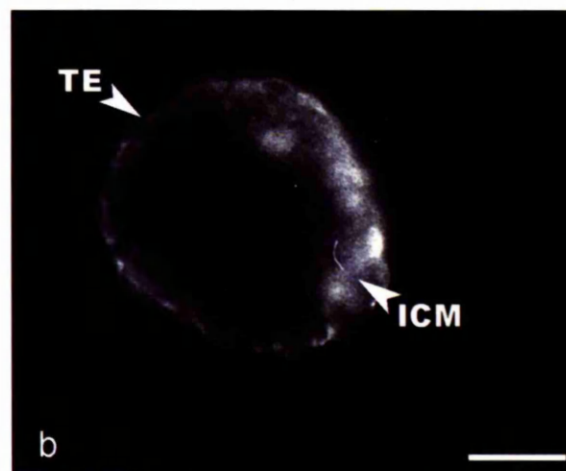
4.2.2.2 αv family integrin expression by mouse blastocysts

Using the antibodies available that cross-react with mouse integrin subunits, αv (AB1930, Figure 4.3b), $\beta 3$ (AB1932, Figure 4.3d) and $\beta 5$ (AB1926, Figure 4.3e, 4.4) were shown to be expressed by mouse blastocysts. Also ten morula stage embryos were stained for $\beta 3$, one of which is shown in Figure 4.3c, demonstrating the punctate distribution of this integrin subunit. The intensity of staining in each case was weak to moderate. Embryos incubated in pre-immune rabbit serum instead of primary antibody were used as negative controls (Figure 4.3f). Optical sectioning of embryos was not possible by standard epifluorescence, but by moving the plane of focus vertically through the embryos it seemed that each integrin subunit was present on both the inner cell mass and trophectoderm. This was further supported by observations of a mouse blastocyst stained for $\beta 5$ that was imaged digitally, from the confocal microscope. Here, cell surface staining is demonstrated in a single z-section (Figure 4.4a), and when the complete z-series is projected as a flat (Figure 4.4b) and 3-D (Figure 4.4c) image. The image in Figure 4.4c is best seen through 3-D glasses.

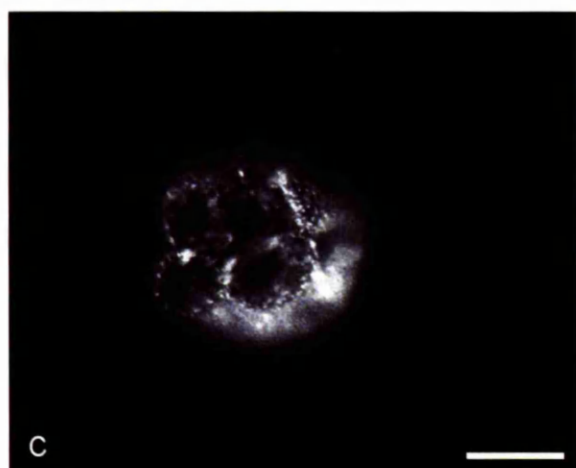
Phase contrast - blastocyst



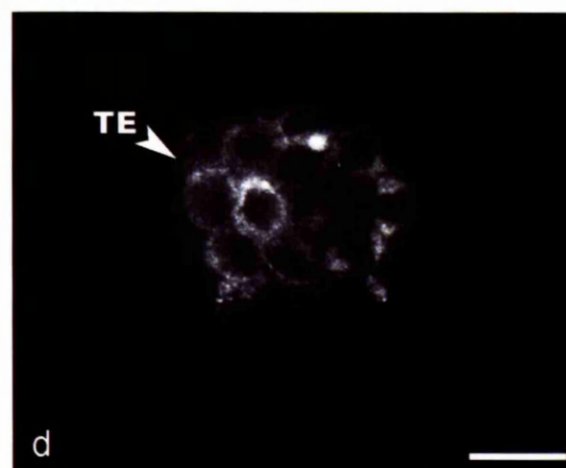
Integrin αv - blastocyst



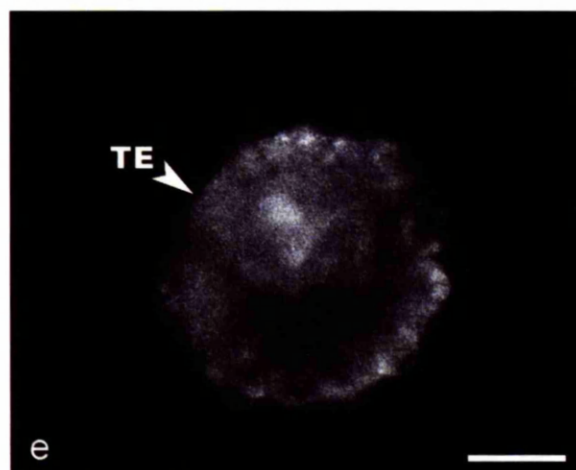
Integrin $\beta 3$ - morula



Integrin $\beta 3$ - blastocyst



Integrin $\beta 5$ - blastocyst



Negative control - blastocyst

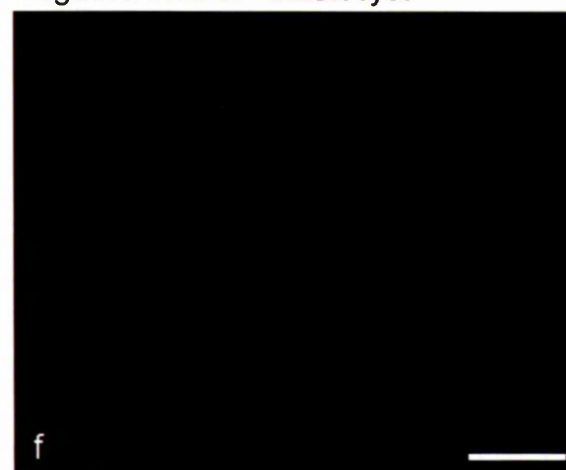


Figure 4.3 Immunolocalisation of αv integrins in mouse embryos

Embryos collected at the 2-cell stage and maintained in vitro to the blastocyst stage

Seen under epifluorescence are a morula (c) and blastocyst stage embryos (b, d-f).

The blastocyst shown by phase contrast (a) is also seen in (b), see 4.2.2.2

TE, trophectoderm epithelium; **ICM**, inner cell mass; scale bar = 50 μm

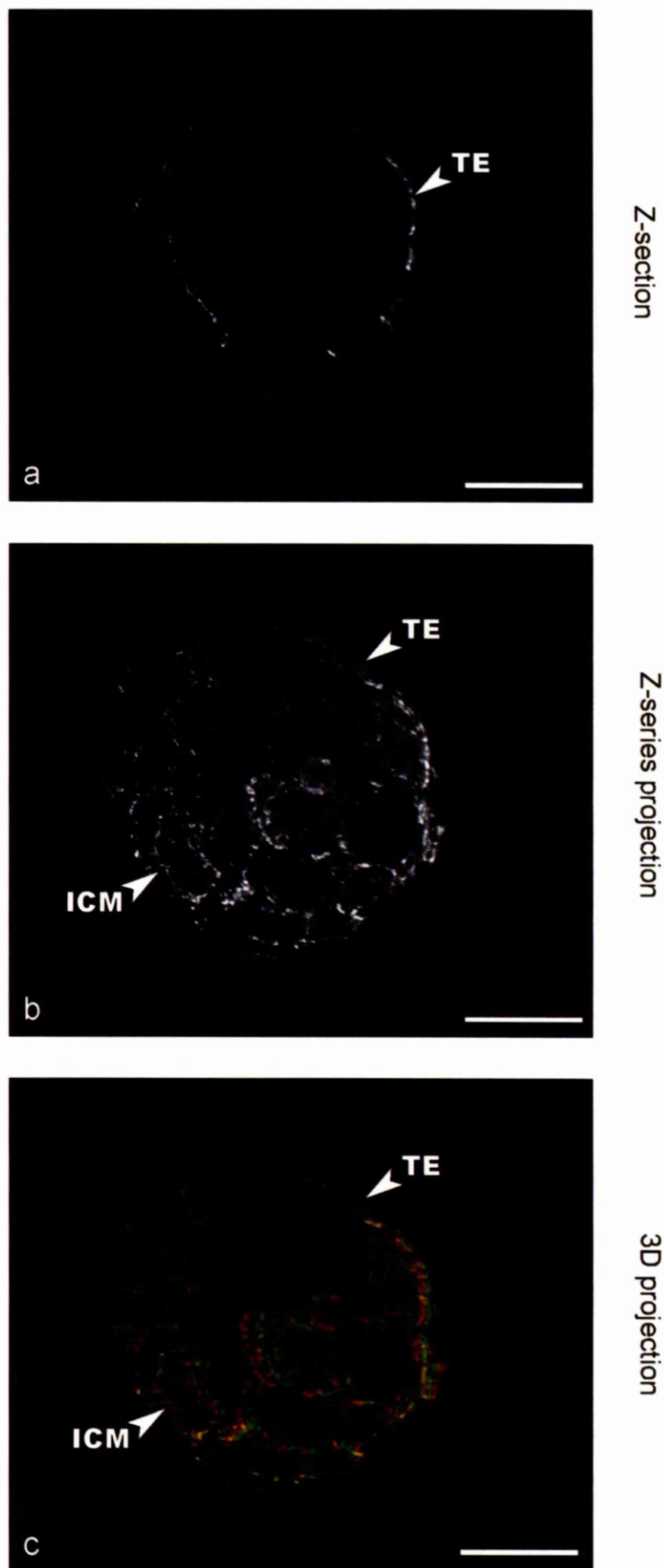


Figure 4.4 Immunolocalisation of $\beta 5$ integrins in a mouse blastocyst

Embryos collected at the 2-cell stage were maintained in vitro to the blastocyst stage. Confocal images collected in Z-plane (a) were compiled, projected flat (b) and in 3D (c). **TE**, trophectoderm epithelium; **ICM**, inner cell mass; see 4.2.2.2, scale bar = 50 μm

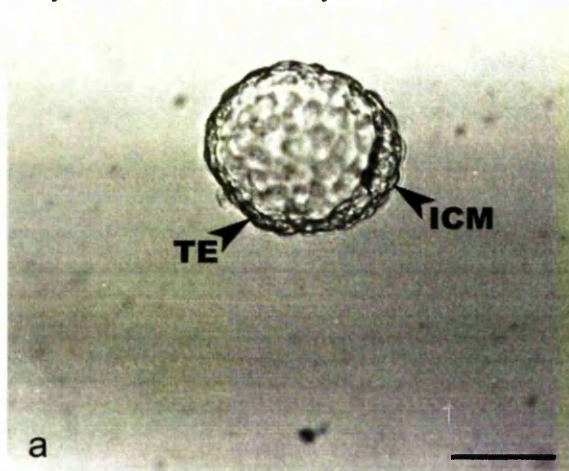
4.2.3 An in vitro model of mouse implantation

In an in vitro model of early trophoblast invasion, hatched mouse blastocysts were transferred into culture medium containing serum (2.1.8). Trophoblast cells spread and migrated on the serum protein substrate. These trophoblast outgrowths were fixed at various stages of development and their integrin profile was examined (2.1.9). Better trophoblast outgrowth was achieved when embryos were cultured on a glass rather than plastic surface.

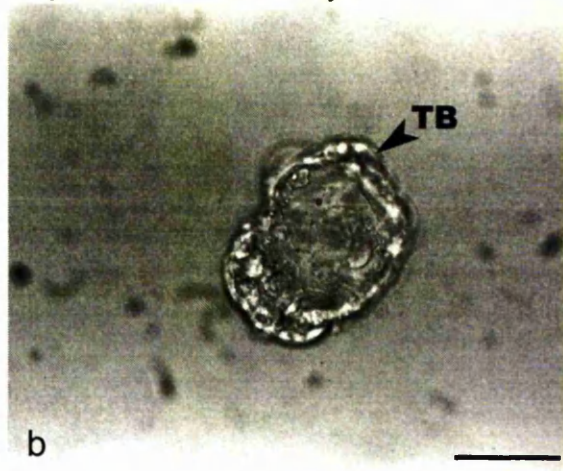
4.2.3.1 Development of trophoblast outgrowths in vitro

On 'day 1 of outgrowth' hatched blastocysts (day of development 6) were transferred to outgrowth culture (section 2.1.8) and rapidly attached to the serum-coated surface (Figure 4.5a). By day 2 the embryos had flattened (Figure 4.5b) and by day 3 the first spread trophoblast cells were recognisable (Figure 4.5c). As the outgrowth developed further, more trophoblast cells were seen outgrowing and these cells became progressively more flattened (Figures 4.5d/e). By day 6 a carpet of highly spread trophoblast cells was seen (Figure 4.5f).

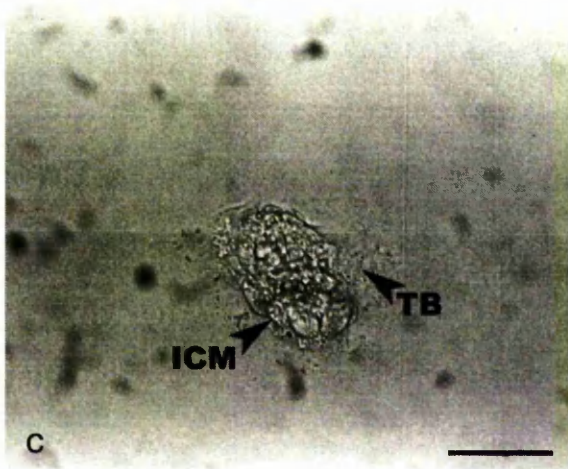
Day 1 attached embryo



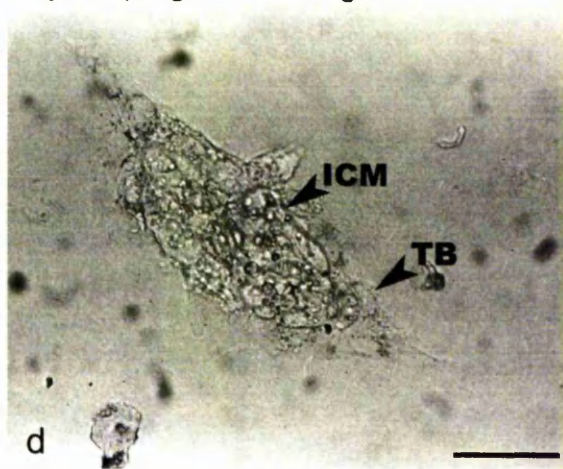
Day 2 flattened embryo



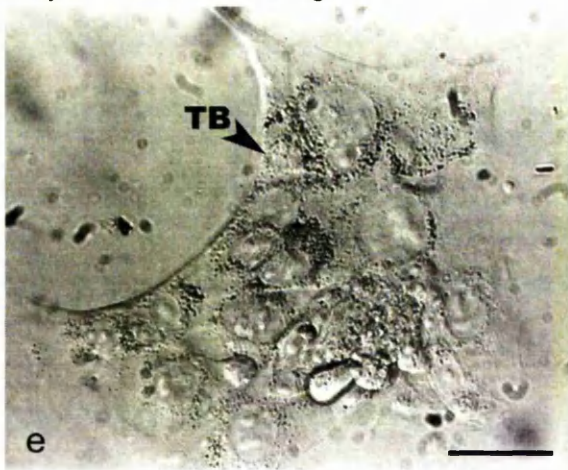
Day 3 early outgrowth



Day 4 progressive outgrowth



Day 5 extensive outgrowth



Day 6 extensive outgrowth

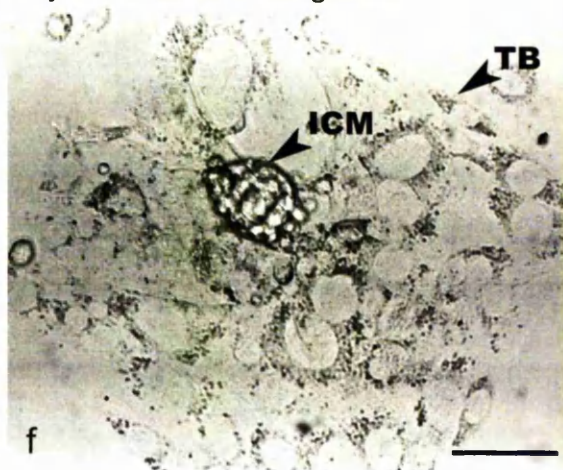


Figure 4.5 Development of trophoblast outgrowths in vitro

Embryos cultured to the blastocyst stage were transferred to serum-containing medium. Trophoblast outgrowths shown by phase contrast microscopy, scale bar = 100 μ m

ICM, inner cell mass; **TE**, trophectoderm; **TB**, outgrowing trophoblasts; see 4.2.3.1

4.2.3.2 αv family integrin expression in trophoblast outgrowths

The expression of the αv , $\beta 1$, $\beta 3$ and $\beta 5$ integrin subunits (using antibodies C8F12, R48, AB1932 and AB1926 respectively) was examined in trophoblast cells on days 3 to 6 of outgrowth (2.1.9). Three distinct patterns of integrin subunit distribution were identified and recorded (Figures 4.6-9; Table 4.2). None of these patterns were characteristic of negative control outgrowths stained with pre-immune rabbit serum instead of primary antibody (Figure 4.9c).

Diffuse cell surface staining

This describes a fine punctate pattern of staining over the entire surface of the trophoblast cell. This distribution was seen for every integrin subunit. It was present from day 3 of outgrowth for αv (Figure 4.6a), $\beta 1$ (Figure 4.7a) and probably $\beta 5$ (Figure 4.9a). Expression of $\beta 3$ in this diffuse pattern was not clear until day 5 of outgrowth (Figure 4.8a).

Dorsal clusters

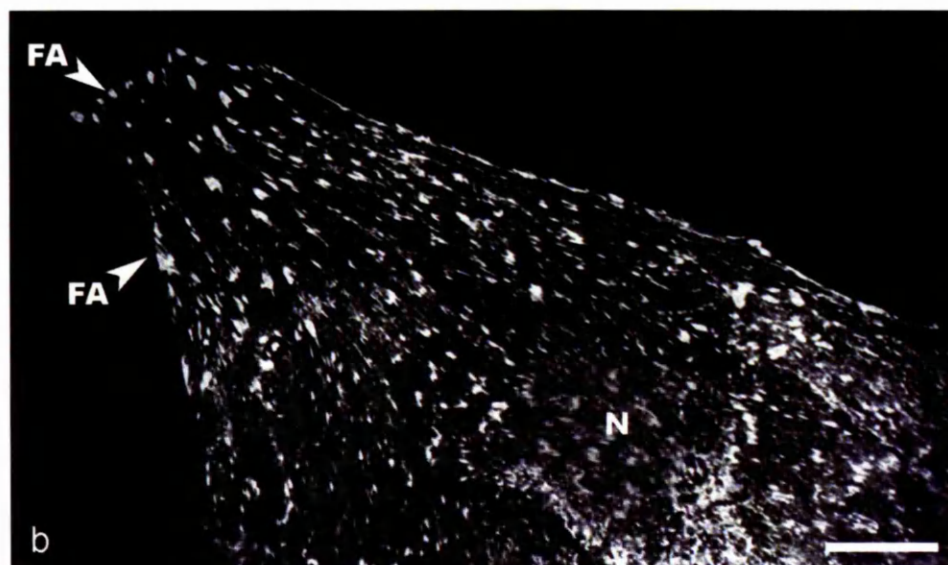
These are larger speckles which appeared to be on the dorsal surface of trophoblasts cells. The clusters contained $\beta 1$ (Figure 4.7b), observed from day 3 of outgrowth. $\beta 5$ -containing clusters were seen from day 4 of outgrowth (Figure 4.9b). At most stages αv was also identified in dorsal clusters, but these were not as obvious or numerous as those containing $\beta 1$ or $\beta 5$. The $\beta 3$ integrin subunit was never identified in dorsal surface clusters.

Focal adhesions

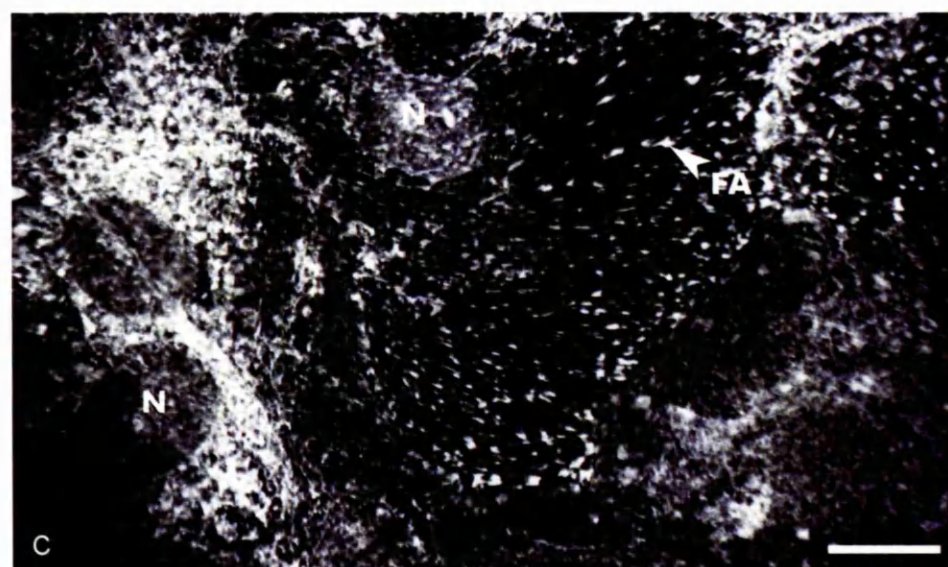
Integrin subunits were also localised to the ventral (substrate-facing) trophoblast cell membrane in a dash-like pattern which is typical of focal adhesions (section 1.7.4). Focal adhesions contained αv (Figure 4.6c/d) and $\beta 3$ (Figure 4.8c/d) at every stage examined. Conversely, $\beta 5$ was never present in focal adhesions (compare Figure 4.9a). $\beta 1$ almost never present in focal adhesions, seen only on day 3 of outgrowth (Figure 4.7c). $\beta 1$ -positive focal adhesions at this stage were rare, and not as striking as those seen containing αv or $\beta 3$.



Diffuse cell surface - day 6



Focal adhesions - day 6



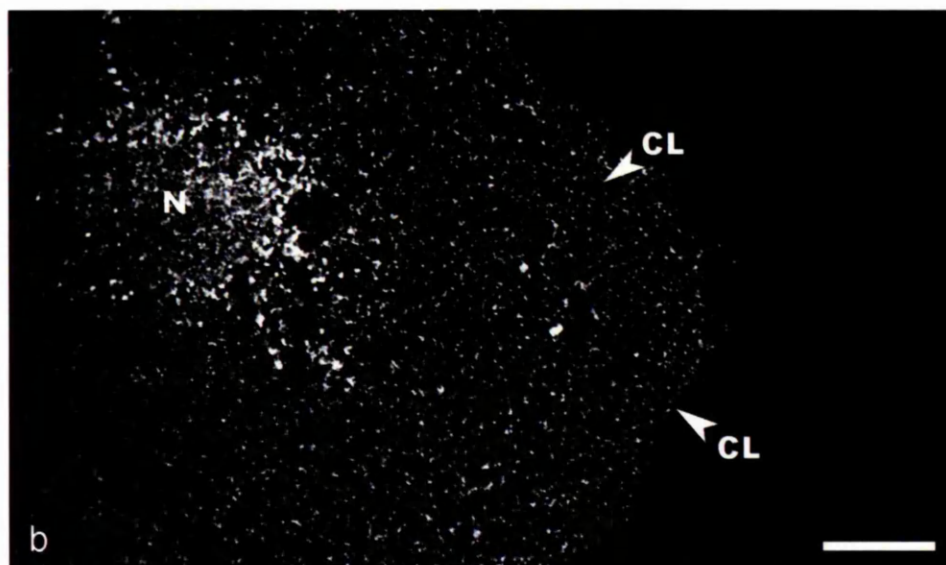
Focal adhesions - day 4

Figure 4.6 Immunolocalisation of α_v integrins in trophoblast outgrowths

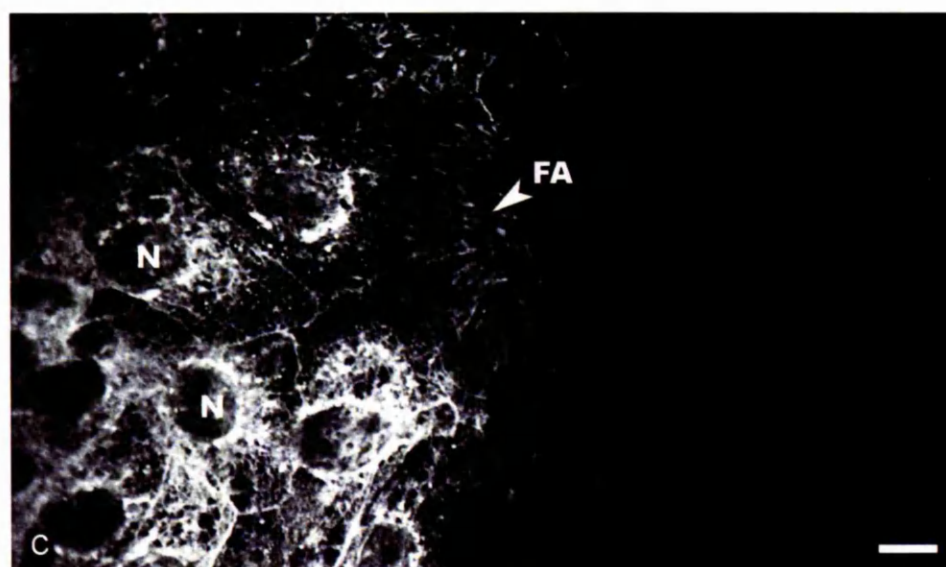
Trophoblast outgrowth on serum proteins. Single digital images (a-c) were collected in the Z-plane, by confocal microscopy, the same cell is shown in (a) and (b), see 4.2.3.2 **P**, punctate stain; **FA**, focal adhesion; **N**, nucleus; scale bar = 25 μ m



Diffuse cell surface - day 5



Dorsal clusters - day 5



Focal adhesions - day 3

Figure 4.7 Immunolocalisation of $\beta 1$ integrins in trophoblast outgrowths

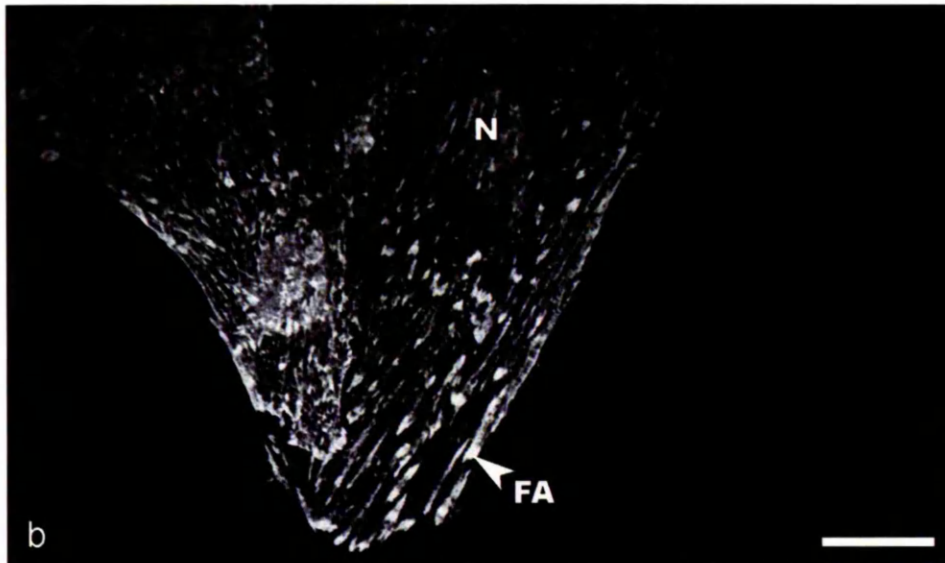
Trophoblast outgrowths were maintained in serum-containing medium (4.2.3.2)

Single digital images (a-c) were collected in the Z-plane, by confocal microscopy

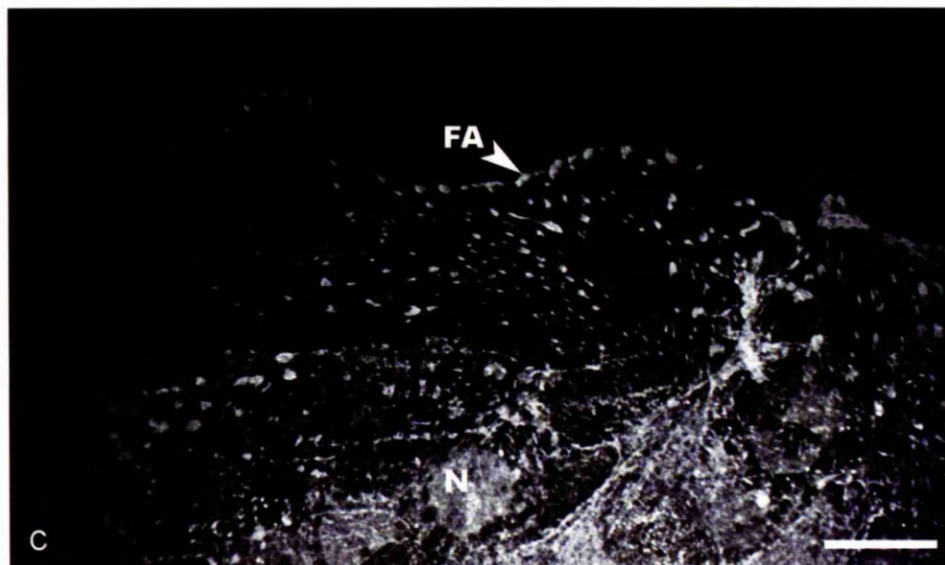
P, punctate; **CL**, integrin clusters; **FA**, focal adhesion; **N**, nucleus; scale bar = 25 μ m



Diffuse cell surface - day 6



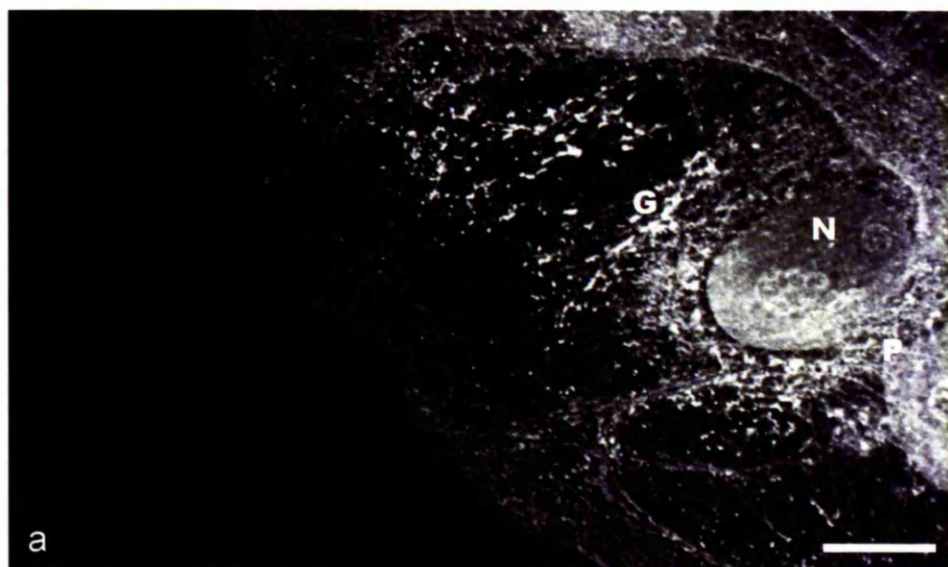
Focal adhesions - day 6



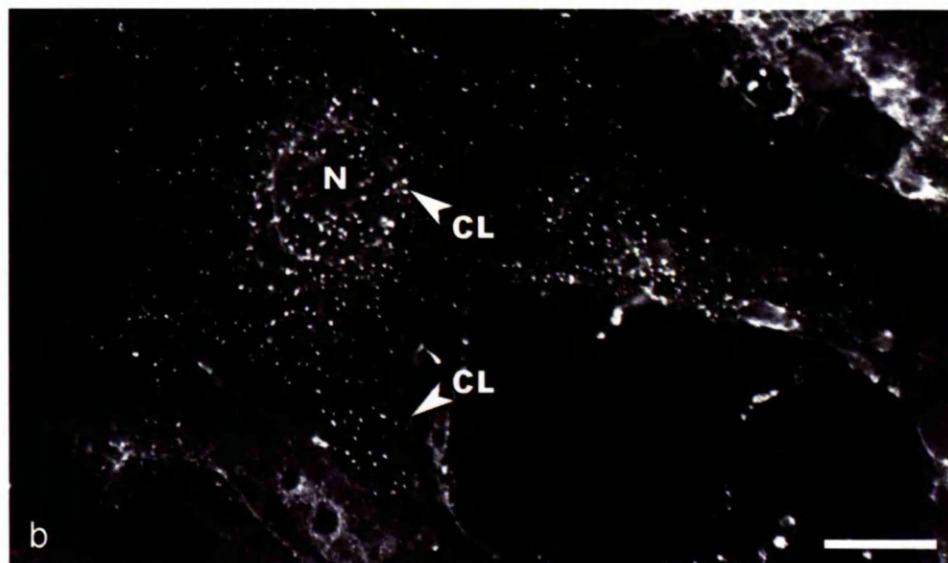
Focal adhesions - day 6

Figure 4.8 Immunolocalisation of $\beta 3$ integrins in trophoblast outgrowths

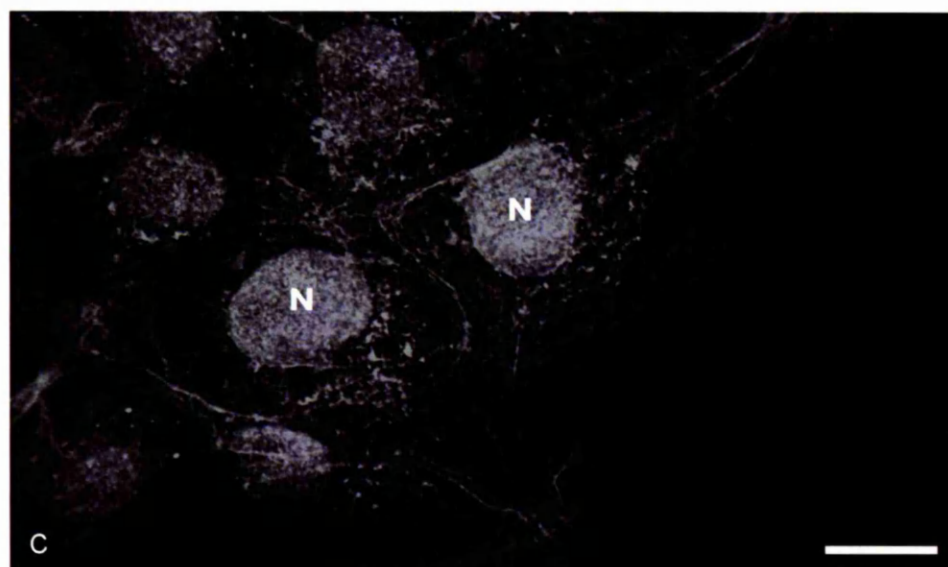
Trophoblast outgrowth on serum proteins. Single digital images (a-c) were collected in the Z-plane, by confocal microscopy, the same cell is shown in (a) and (b), see 4.2.3.2 **P**, punctate stain; **FA**, focal adhesion; **N**, nucleus; scale bar = 25 μ m



Diffuse cell surface - day 6



Dorsal clusters - day 5



Negative control - day 6

Figure 4.9 Immunolocalisation of $\beta 5$ integrins in trophoblast outgrowths

Trophoblast outgrowths were maintained in serum-containing medium (4.2.3.2). Single digital images (a-c) were collected in the Z-plane, by confocal microscopy. **P**, punctate; **CL**, integrin clusters; **G**, granules; **N**, nucleus; scale bar = 25 μ m

Table 4.2 α v family integrin expression in trophoblast outgrowths

| Pattern of staining | Day of trophoblast outgrowth | | | |
|-----------------------------|------------------------------|-------|-------|-------|
| | Day 3 | Day 4 | Day 5 | Day 6 |
| α v | | | | |
| <i>Diffuse cell surface</i> | + | + | + | + |
| <i>Dorsal clusters</i> | (+) | (+) | (+) | — |
| <i>Focal adhesion</i> | (+) | + | + | + |
| β 1 | | | | |
| <i>Diffuse cell surface</i> | + | + | + | + |
| <i>Dorsal clusters</i> | (+) | + | + | + |
| <i>Focal adhesion</i> | (+) | — | — | — |
| β 3 | | | | |
| <i>Diffuse cell surface</i> | — | — | + | + |
| <i>Dorsal clusters</i> | — | — | — | — |
| <i>Focal adhesion</i> | (+) | + | + | + |
| β 5 | | | | |
| <i>Diffuse cell surface</i> | (+) | + | + | + |
| <i>Dorsal clusters</i> | — | (+) | + | + |
| <i>Focal adhesion</i> | — | — | — | — |

[+, most often present; (+), occasionally present; —, not observed]

4.3 DISCUSSION

Integrins and other cell adhesion molecules have been demonstrated by immunofluorescence in pre-implantation human embryos (Campbell *et al.*, 1995b). Here, consistent expression of $\alpha 3$, αv , $\beta 1$, $\beta 3$, $\beta 4$ and $\beta 5$ was seen from oocytes to morulae or blastocysts (where available), in spite of the changes in cellular interaction which accompany pre-implantation development. To further this work I used a rarely available source of morphologically normal human blastocysts and showed the presence of the αv , $\beta 1$, $\beta 3$ and $\beta 5$ integrin subunits predominantly at the trophoblast layer, providing further evidence for a role for these molecules in implantation.

In the mouse, the presence of mRNA and protein of subunits $\alpha 5$, $\alpha 6B$, αv , $\beta 1$ and $\beta 3$ has been shown throughout pre-implantation development and appears unregulated (Sutherland *et al.*, 1993). Several other integrin subunits, including $\alpha 1$, $\alpha 2$, $\alpha 6A$ and $\alpha 7$ are developmentally regulated, where expression coincides with the onset of differentiation of extraembryonic lineages (Sutherland *et al.*, 1993). In the present study, expression of αv , $\beta 3$ and $\beta 5$ (Spanswick *et al.*, 1995) was also demonstrated in mouse blastocysts, and $\beta 3$ was identified in some morulae. These integrin subunits were localised to the external surface of embryos, at the trophectoderm surface in blastocysts. With blastocoel formation, the $\alpha v\beta 3$ dimer has been demonstrated predominantly at this location, while $\alpha 5\beta 1$ and $\alpha 6\beta 1$ become restricted to the ICM (Sutherland *et al.*, 1993). Therefore several integrins are potentially important throughout pre-implantation embryo development, and αv family integrins are suitably located to mediate initial trophoblast interactions at implantation in both mouse and human.

Integrins on the trophectoderm may bind several ECM ligands that are present at this surface. LM and oncofetal FN have been identified on human pre-implantation embryos (Turpeenniemi-Hujanen *et al.*, 1992, 1995), and in mouse blastocysts, LM and TSP have been localised to the trophectoderm surface (O'Shea *et al.*, 1990; Dziadek & Timpl, 1985). These proteins may bind receptors on the endometrial surface, thereby facilitating embryo attachment. Null mutation

of the mouse gene encoding LM γ 1 chain present in most forms of LM leads to failure of implantation at around day 6.5; therefore in the mouse LM may be more important in the stages following initial attachment (Dr D Edgar, personal communication). Also embryonic integrins may interact directly with components of the apical endometrial epithelium, such as OST (Brown *et al.*, 1992). Ligation of embryonic integrins may induce intracellular signalling events that are required to promote embryonic development beyond the blastocyst stage, and thereby allow the implantation process to advance. Following embryo attachment, trophoblast integrins could mediate trophoblast invasion. During invasion, trophoblasts interact with an array of ECM components, present at the uterine epithelial basement membrane and within the endometrial stroma (section 1.8.1). For example, the oncofetal form of FN has been localised to the points of contact between trophoblast and decidual cells (Feinberg *et al.*, 1991).

During human placentation, progressive changes in CTB integrin expression occur with advancing invasion, reflecting alterations in the uterine extracellular environment. These changes include loss of $\alpha v\beta 5$, but not $\alpha v\beta 3$, with CTB migration (Damsky *et al.*, 1994, 1997). These two closely related integrins, differentially regulated following initial implantation, appear to have distinct functions. Expression of αv family integrins following embryo attachment, was studied in a model of early invasion in the mouse. Mouse embryo outgrowth *in vitro* may be analogous to the early phases of implantation *in utero* (Hsu, 1971). In simple medium supplemented with heat inactivated fetal calf serum (10%), trophoblast cells outgrow from blastocysts as a monolayer, while the ICM initially remains as a solid clump of cells. Following approximately 10 days of outgrowth the ICM of a proportion of embryos will have spread on top of the trophoblast outgrowth, but up to this stage it is generally accepted that the population of migratory cells which outgrows is of the trophoblast lineage (Sherman, 1975). In medium supplemented with $\geq 3\%$ serum, VN is the protein responsible for supporting cell adhesion (Knox, 1984).

This investigation demonstrated several distinct patterns of integrin distribution in outgrowing trophoblasts. These highlight integrin-specific changes which must occur with transition from blastocyst trophectoderm, where all αv family integrins share a similar distribution, to outgrowing trophoblast. In outgrowths a fine punctate staining pattern, as seen for all subunits examined, has been previously observed when trophoblasts outgrow on FN and are stained with antibodies to αv , $\alpha 11b$ and $\beta 3$. On FN, $\alpha 3$, $\alpha 5$, αv , $\beta 1$ and $\beta 3$ localise to focal adhesions, suggesting the presence of integrins $\alpha 3\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 3$ (Yelian *et al.*, 1995). However on VN, $\beta 1$ was very rarely observed at focal adhesions and was largely present in a fine punctate pattern. $\alpha v\beta 3$ appeared to be the main αv family integrin in focal adhesions, while $\alpha v\beta 5$ was excluded from these structures.

Previous studies have demonstrated that, organisation of $\alpha v\beta 3$ and $\alpha v\beta 5$ is regulated by divalent cations (in an osteosarcoma cell line), where focal adhesions containing $\alpha v\beta 3$ formed in the presence Mg^{2+} or Mn^{2+} , but not Ca^{2+} . The same cells also express $\alpha v\beta 5$, this localises to focal adhesions with Mg^{2+} or Ca^{2+} , but not Mn^{2+} (Stuiver *et al.*, 1996). M16 medium (Sigma), in which serum-dependent outgrowths are cultured, contains salts of Mg^{2+} and Ca^{2+} but not Mn^{2+} , so in outgrowths it may be the formulation of the medium which dictates the localisation of αv family integrins. This could be tested using alternative formulations of culture medium. It has also been shown, using integrin $\beta 1/\beta 5$ chimeras, that the cytoplasmic tail directs association with focal adhesions (Pasqualini & Hemler, 1994). Therefore 'outside-in' transduction of information may be occurring through integrins in trophoblast outgrowths.

The presence of $\beta 1$ and $\beta 5$ in dorsal surface clusters has not been described previously and the function of integrins in this location is not clear. Integrin clusters on the dorsal surface may mediate VN internalisation by trophoblasts. Previously it has been shown that human skin fibroblasts use $\beta 5$ integrins alone for VN endocytosis, and that this could be blocked by anti- $\alpha v\beta 5$ antibodies or protein kinase C inhibitors (Panetti & McKeown-Longo, 1993; Panetti *et al.*, 1995). During internalisation, VN and $\beta 5$ are co-localised in endocytotic vesicles

(Memmo & McKeown-Longo, 1998). Similarly, astrocytoma cells on a range of substrates can internalise and degrade soluble VN via either $\alpha_v\beta_3$ or $\alpha_v\beta_5$. Here, the importance of either integrin is dependent on the substrate to which the cells are attached (Pijuan-Thompson & Gladson, 1997). As there was a more limited expression of α_v in dorsal clusters of trophoblasts, this perhaps provides further evidence for an alternative partner for β_5 (as discussed in Chapter 3) or β_1 at this location.

Integrin expression (and perhaps distribution) by trophoblasts is at least in part dictated by the ligand availability (Schultz & Armant, 1996). Therefore it was of great interest to examine whether integrins became differentially localised when trophoblasts were outgrown on other substrates (in the absence of serum). However, following repeated attempts I was unable to localise any integrin subunits in outgrowing trophoblasts cultured in the absence of serum. It is not clear why immunostaining of serum-free outgrowths was unsuccessful. I did explore the possibility there may have been a sensitivity problem by using three-step immunofluorescence (2.1.3) with little success. It is possible that in the absence of serum, the cytoskeleton and associated proteins (including integrins) do not become as highly organised. Also, in the presence of serum the best outgrowth immunostaining was achieved when embryos were at later stages of outgrowth (days 5 and 6) but serum-free outgrowths are viable for less time, cultured until day 4 only. As an alternative to immunostaining and to further study integrins in trophoblast outgrowth, function blocking antibodies against α_v and β_1 were instead added to outgrowth cultures, as described in Chapter 5.

CHAPTER 5

An investigation of integrin function in implantation

5.1 INTRODUCTION

Having established the expression pattern of αv family integrin subunits in uterine and embryonic tissues, further work was undertaken to examine the roles of these molecules in implantation. This study concentrated on the αv and $\beta 1$ integrin subunits, to determine the relative importance of these two families of integrins in an in vitro model of implantation in which trophoblast cells outgrow on specific extracellular matrix substrates. The ability of several integrin ligands to support trophoblast outgrowth was studied. Function blocking antibodies to integrins αv and $\beta 1$ determined the role of these subunits, expressed by trophoblast cells, in mediating outgrowth. A natural progression from these in vitro experiments was to use function-blocking antibodies in vivo. Little data regarding in vivo investigation of implantation is available, although an intra-uterine injection technique in mice has been published, suggesting that a preliminary investigation using anti-integrin antibodies might be feasible.

5.2 RESULTS

Part 1 - Integrin function in an in vitro model of mouse implantation

5.2.1 Optimisation of outgrowth cultures

Culture of embryos in the presence of serum (4.2.3.1) provides a mixed substrate for trophoblast cell migration owing to the adsorption of serum proteins to the culture dish. To examine the function of specific integrin subunits on identified substrates, outgrowths were maintained in the absence of serum. A published method for maintaining embryo outgrowths in serum-free conditions on a protein coated surface was adapted for the purpose of this research. Several features of this new method (section 2.1.10) were shown to be very important for maintaining the viability of embryos developing beyond the blastocyst stage, and for promoting trophoblast outgrowth in the absence of serum. The method required hatched blastocysts. These were cultured in small volumes (100 μ l) in groups of at least 10 (optimally 15-20) for up to 72 hours. The use of 8-well chamber slides (Figure 2.1c) made pre-coating and blocking the glass surface simple. By this method, a range of substrates or culture conditions could be tested using just one or two slides. Coating the substrate onto a glass rather than

a plastic chamber slide was necessary to produce good trophoblast outgrowth. The time at which outgrowth culture was initiated was designated 'day 1' and the final stage of outgrowth (after culture for 72 hours) was termed 'day 4'.

5.2.2 Assessment and classification of outgrowth stage

In order for the trophoblast cells of a blastocyst to outgrow on a given substrate, the embryo must first attach. Under control conditions this occurs by day 2 of outgrowth culture. Trophoblast outgrowth was not normally evident before day 3. Cultures maintained past day 4, in the absence of serum, began to show signs of degeneration. Therefore data was collected from cultures at days 3 and 4 of outgrowth. Three distinct stages were used to classify the development of outgrowths:

Stage 1 - Unattached

The embryo could be seen to be floating or rolling on gentle agitation of the slide.

Stage 2 - Attached

At this stage the embryo had firmly attached to the substrate and usually appeared more flattened. On microscopic examination, no trophoblast cells were seen spreading or migrating away from the main mass of the embryo.

Stage 3 - Outgrowing

Here, trophoblast cells were seen spreading from the main mass of the embryo. Typically this described extensive outgrowth, where numerous trophoblast cells had extended laterally from the embryo. Also included in this group were a few embryos where just one or two outgrowing cells were seen.

The raw data generated in outgrowth experiments, showing actual numbers of embryos seen at each stage of outgrowth on different substrates under various conditions are shown in Appendix 4.1.

5.2.3 Presentation and analysis of data from outgrowth studies

For simple comparison of the efficacy of each substrate/condition in supporting outgrowth, the percentages of embryos at each stage, that is unattached, attached and outgrowing were calculated and plotted as horizontal bar charts. For further analysis embryos were split into two groups, showing outgrowth or non-outgrowth (unattached and attached). The statistical package SIMFIT (W. Bardsley, University of Manchester) was used to assist with statistical analyses. A chi-squared (χ^2) test was used to calculate statistical significance of differences seen between proportions of embryos showing outgrowth ('success') or non-outgrowth ('failure') in a range of conditions. Three levels of significance were used where, $p \leq 0.001$ (denoted ***), $p \leq 0.005$ (denoted **) and $p \leq 0.01$ (denoted *). No significant difference was indicated when $p > 0.01$ (denoted NS). Finally, the proportions of embryos outgrowing ('success') out of the total number of embryos ('total') was plotted on vertical bar charts, with upper and lower confidence limits (at 95%) shown as error bars.

5.2.4 The ability of various substrates to support trophoblast outgrowth

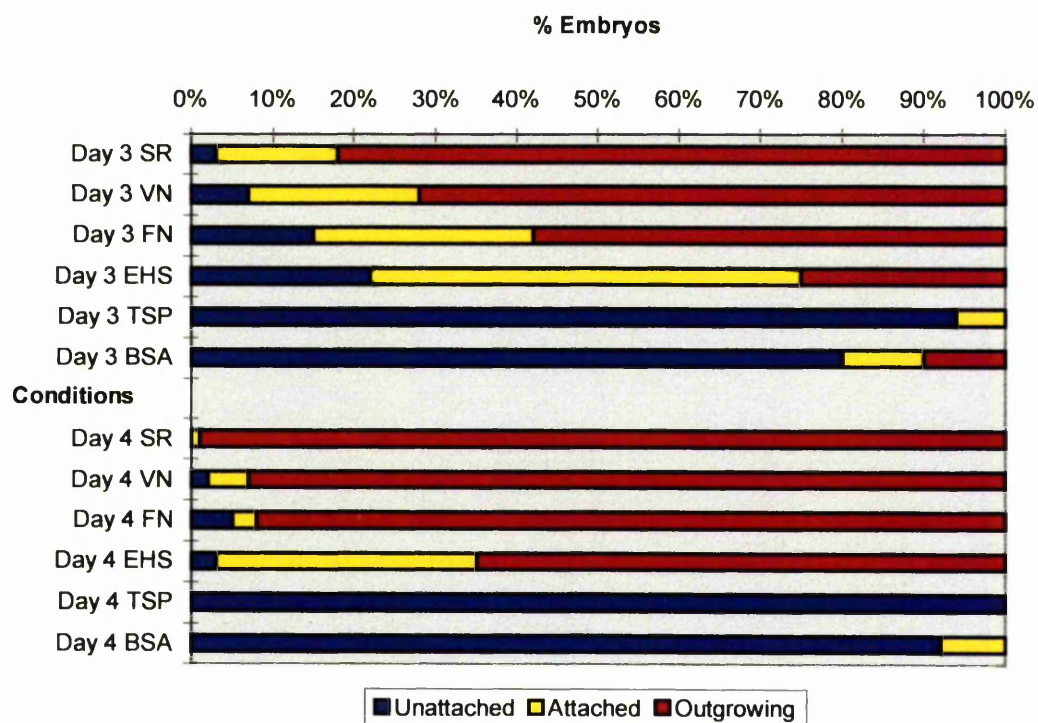
5.2.4.1 The effect of substrate type on stage of outgrowth achieved

Initial experiments to determine the ability of individual substrates to support embryo outgrowth in the absence of serum proteins (2.1.10) were carried out and the results were compared to embryos cultured in serum-containing medium (2.1.8). VN, FN and TSP are αv family integrin ligands. EHS matrix, containing LM and COL IV, was used as a $\beta 1$ integrin family ligand and BSA was used as a negative control substrate.

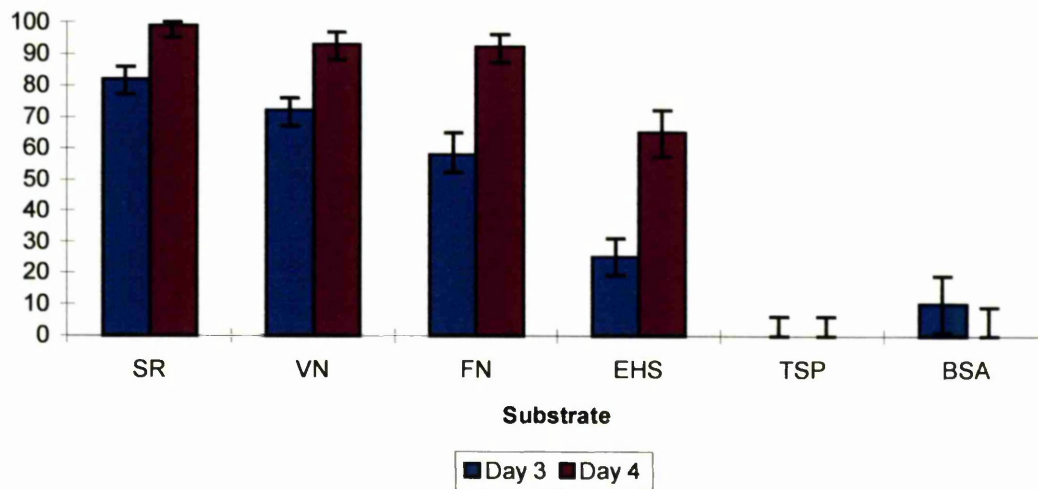
On day 3 embryos cultured in the presence of serum were the most advanced, with the greatest proportion of embryos outgrowing and the fewest unattached (Figure 5.1). The proportion of embryos outgrowing on VN, FN or EHS substrates were all significantly different to that seen in the presence of serum. Furthermore, the time before outgrowth was initiated on each of these substrates was significantly different to that seen on the other substrates (Figure 5.2, Table 5.1). Therefore, by day 3, embryos on VN, FN or EHS each showed distinct outgrowth

profiles and the proportion of embryos outgrowing on each substrate was significantly different to that seen on BSA. TSP did not support embryo outgrowth (Figures 5.1, 5.2). This phenomenon was not due to an inability of TSP to coat the slide (5.2.4.2). BSA was used as a negative control substrate and also to block non-specific binding to all other substrates. A few embryos were seen outgrowing on BSA, although in every case only one or two trophoblast cells were spreading from the embryo. There was no significant difference between the proportion of embryos either attached or outgrowing on TSP or BSA (Figure 5.1, Table 5.1).

By day 4 the proportion of embryos outgrowing on serum, VN, FN or EHS matrix had increased significantly from that seen on day 3. Also, by day 4 the numbers of embryos at each stage of outgrowth on VN or FN substrates were comparable to those cultured in the presence of serum (Figure 5.1). There was no significant difference between the proportions of outgrowing embryos on VN, FN or serum. This demonstrated that maintaining outgrowths under serum-free conditions was as effective as serum-dependent cultures; and showed that outgrowth success on VN or FN were similar (Figure 5.2, Table 5.1). The proportion of embryos outgrowing on EHS matrix on day 4 was significantly less than that seen on either VN, FN or in the presence of serum at the equivalent stage. However, the proportion of embryos outgrowing on EHS matrix on day 4 was not significantly different to that seen on either VN or FN substrates on day 3 of outgrowth (Figure 5.2, Table 5.1). Therefore it seemed that although EHS could adequately support embryo outgrowth, this process was delayed when compared to embryos on other substrates under otherwise identical culture conditions. No embryos were outgrowing on TSP or BSA on day 4 (Figure 5.1) and there was no significant difference between the proportion of embryos attached to these two substrates. The absence of outgrowth on TSP was irrespective of whether calcium was present during adsorption of this substrate to the slide (2.1.10.1). It appeared that the few embryos showing limited trophoblast outgrowth on BSA on day 3 were not able to maintain these spread cells by day 4. It is not clear whether these embryos remained attached to BSA or detached.

Figure 5.1 Effect of substrate on trophoblast outgrowth development

[SR, serum; VN, vitronectin; FN, fibronectin; EHS, EHS matrix; TSP, thrombospondin]

Figure 5.2 Effect of substrate type on proportion of embryos outgrowing**% Embryos outgrowing**

See Table 5.1 for statistical analysis

[SR, serum; VN, vitronectin; FN, fibronectin; EHS, EHS matrix; TSP, thrombospondin]

Table 5.1 Successful embryo outgrowth - significance of substrate type

| Comparison of outgrowth success | χ^2 test | Level of |
|---|---------------|--------------|
| | p value | significance |
| day 3 serum vs. day 3 vitronectin | = 0.0033 | ** |
| day 3 serum vs. day 3 fibronectin | \leq 0.0001 | *** |
| day 3 serum vs. day 3 EHS matrix | \leq 0.0001 | *** |
| day 3 vitronectin vs. day 3 fibronectin | = 0.0006 | *** |
| day 3 vitronectin vs. day 3 EHS matrix | \leq 0.0001 | *** |
| day 3 fibronectin vs. day 3 EHS matrix | \leq 0.0001 | *** |
| day 3 fibronectin vs. day 4 fibronectin | \leq 0.0001 | *** |
| day 3 vitronectin vs. day 4 vitronectin | \leq 0.0001 | *** |
| day 3 EHS matrix vs. day 4 EHS matrix | \leq 0.0001 | *** |
| day 3 BSA vs. day 3 vitronectin | \leq 0.0001 | *** |
| day 3 BSA vs. day 3 fibronectin | \leq 0.0001 | *** |
| day 3 BSA vs. day 3 EHS matrix | = 0.0083 | * |
| day 3 BSA vs. day 3 thrombospondin | = 0.0285 | NS |
| day 3 BSA vs. day 3 thrombospondin ^a | = 0.4104 | NS |
| day 4 serum vs. day 4 vitronectin | = 0.0414 | NS |
| day 4 serum vs. day 4 fibronectin | = 0.0188 | NS |
| day 4 serum vs. day 4 EHS matrix | \leq 0.0001 | *** |
| day 4 vitronectin vs. day 4 fibronectin | = 0.9139 | NS |
| day 4 vitronectin vs. day 4 EHS matrix | \leq 0.0001 | *** |
| day 4 fibronectin vs. day 4 EHS matrix | \leq 0.0001 | *** |
| day 3 fibronectin vs. day 4 EHS matrix | = 0.2227 | NS |
| day 3 vitronectin vs. day 4 EHS matrix | = 0.1190 | NS |
| day 3 serum vs. day 4 serum | \leq 0.0001 | *** |
| day 4 BSA vs. day 4 vitronectin | \leq 0.0001 | *** |
| day 4 BSA vs. day 4 fibronectin | \leq 0.0001 | *** |
| day 4 BSA vs. day 4 EHS matrix | \leq 0.0001 | *** |
| day 4 BSA vs. day 4 thrombospondin | E | NS |
| day 4 BSA vs. day 4 thrombospondin ^a | = 0.9304 | NS |

[^a, attached; NS, non-significant; E, no successes; ***, $p \leq 0.001$; **, $p \leq 0.005$; *, $p \leq 0.01$]

5.2.4.2 Confirmation of thrombospondin substrate coating

As no outgrowth was seen on TSP it was necessary to confirm that this substrate had coated the glass surface of the culture wells. Antibodies to TSP or FN were added to wells coated with the appropriate substrate or control (BSA), this was followed by an incubation with peroxidase conjugated secondary antibody and chemiluminescent detection (section 2.1.10.3; Figure 5.3a). The slide was then exposed to photographic film to produce the image shown in Figure 5.3b. This experiment demonstrated that sufficient TSP had coated the well to enable detection; proving that the lack of embryo outgrowth on TSP was not due to an inability of TSP to adsorb.

5.2.5 Production and purification of an α_v function blocking antibody

Conditioned medium was collected from the C8F12 rat hybridoma clone (Charles Streuli, University of Manchester). A function blocking antibody raised to mouse α_v integrin subunit was purified from the culture supernatant (2.1.13) and is shown by SDS-PAGE under reducing and non-reducing conditions in Figure 5.4. The mobility of C8F12 purified proteins was as expected for rat. Purified antibody was concentrated to 2mg/ml IgG.

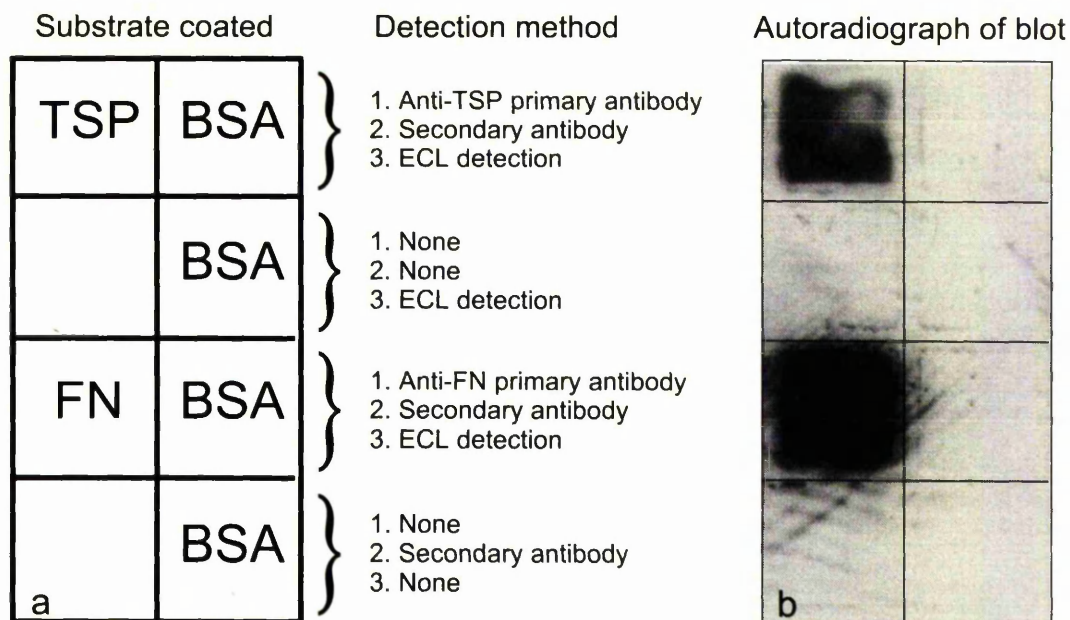


Figure 5.3 Immunodetection of thrombospondin to confirm coating

Substrates were coated onto a glass slide (a) and exposed to photographic film (b), following immunodetection. Several controls demonstrated specific detection (5.2.4.2)

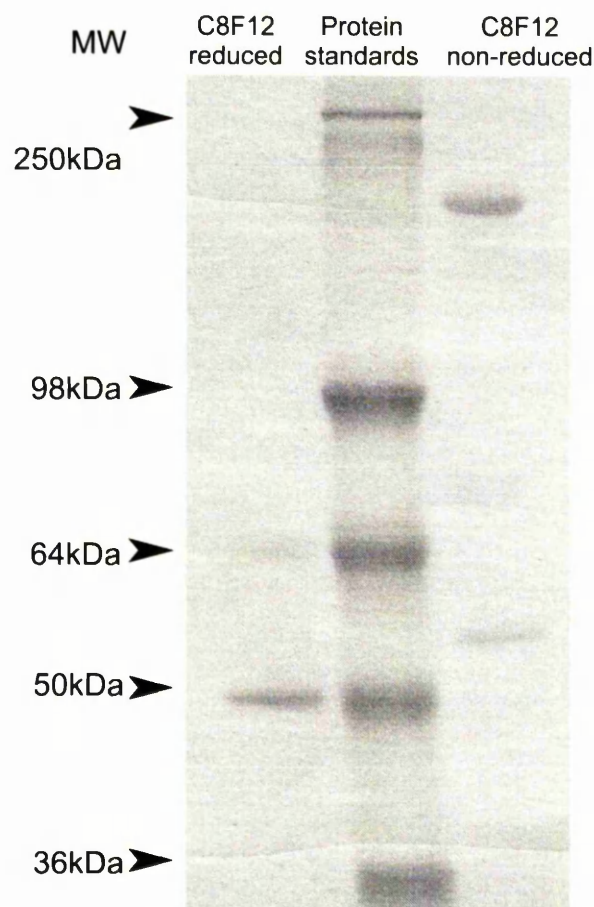


Figure 5.4 Anti- α_v antibody purified from C8F12 hybridoma medium

C8F12 culture supernatant was passed through a protein G sepharose column. Purification was confirmed by separation of the column eluate by acrylamide gel electrophoresis, and Coomassie staining of the gel. See section 5.2.5.

5.2.6 Determination of antibody dose required to inhibit outgrowth

Prior to functional studies using anti- α v integrin (C8F12) and anti- β 1 integrin (R48) antibodies it was necessary to determine the concentration at which they were effective. The anti- α v function blocking antibody (stock 2mg/ml) was added to serum-containing culture medium to produce a range of dilutions (1:5 to 1:100) with final IgG concentrations of 20-400 μ g/ml. Blastocysts were transferred to this medium and the numbers of outgrowing embryos were counted on days 3 and 4.

On day 3, anti- α v at 200 μ g/ml had reduced the numbers of outgrowths to less than half that seen without antibody (Appendix 4.2). On day 4 no outgrowths were seen in the presence of 200 μ g/ml anti- α v, although over 50% of the control outgrowth rate was seen with 100 μ g/ml of antibody (Appendix 4.2). Therefore, for the purpose of outgrowth blocking studies anti- α v was used at 200 μ g/ml, the lowest concentration at which outgrowth was reduced by over 50% on both days 3 and 4 of outgrowth.

The rabbit polyclonal β 1 subunit blocking antibody R48 (affinity purified; stock 13mg/ml; courtesy of Gwyneth Edwards, University of Manchester) was reported to have function blocking activity at 500-1000 μ g/ml. It was diluted 1:20 or 1:10 (to 650 μ g/ml or 1300 μ g/ml) in serum-free outgrowth medium and added to blastocysts cultured on EHS substrate. No outgrowths were seen on either day 3 or 4, in contrast to embryos cultured in medium without antibody (Appendix 4.2). In further blocking studies anti- β 1 was added to cultures to produce a final IgG concentration of 650 μ g/ml.

5.2.7 The α v and β 1 integrin subunits support embryo outgrowth

Blastocysts were cultured in serum-free conditions (2.1.10) on VN, FN or EHS matrix substrates, in the presence of anti- α v, anti- β 1, both antibodies, or no antibody. As an additional control for the anti- α v antibody, rat IgG was added to cultures at 200 μ g/ml, this did not reduce embryo outgrowth on any substrate. Chi-squared analysis of the effect of culture condition versus substrate are shown in Appendices 4.3 and 4.4.

5.2.7.1 Vitronectin substrate

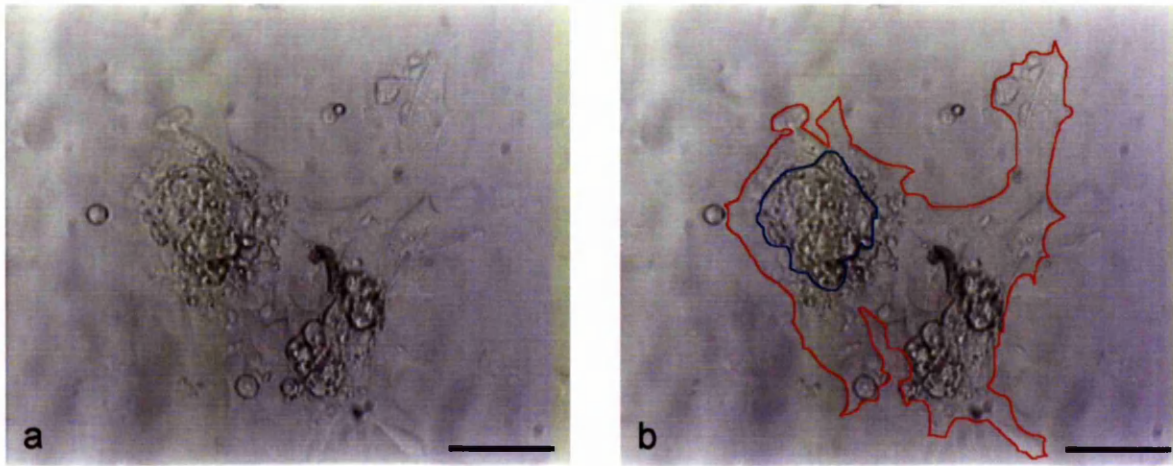
A VN substrate supported embryo outgrowth (5.2.4.1), this was evident for most embryos (72%) by day 3 and confirmed for 93% of embryos by day 4 (Figures 5.1, 5.2, 5.5a/b). Embryos showed a typical pattern of outgrowth, with an expanse of trophoblast cells spreading laterally from the now flattened inner cell mass.

In the presence of anti- α v trophoblast outgrowth was rarely seen (Figure 5.5c/d). Many embryos remained unattached (Figure 5.6) and outgrowth success was significantly lower than in controls (Figure 5.7, Table 5.2). When anti- α v was added alone there was a significant increase in outgrowth between days 3 and 4, although the percentage of outgrowing embryos statistically remained low, at 17% on day 3 and 43% on day 4. This indicated that anti- α v did not completely halt the progression of embryo outgrowth, although it did greatly reduce outgrowth success.

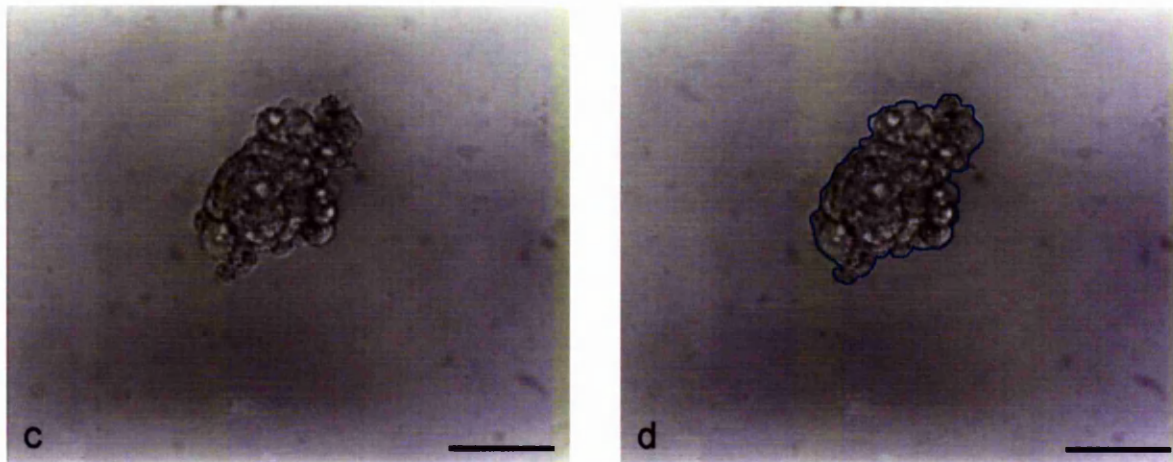
With anti- β 1, outgrowth morphology was largely the same as in controls, although rounded (not spreading) migratory trophoblast cells were also apparent (Figure 5.5e/f). The proportions of embryos at each stage of outgrowth was similar between cultures with anti- β 1 and the controls (Figure 5.6). Addition of anti- β 1 to cultures had no significant effect on outgrowth success (Figure 5.7, Table 5.2).

Addition of anti- α v in combination with anti- β 1 to cultures showed no significant difference in the extent of outgrowth inhibition to that seen with anti- α v alone (Figure 5.6). However unlike with anti- α v alone, addition of both antibodies prevented any significant progression in outgrowth from day 3 to day 4 of culture (Figure 5.7, Table 5.2).

Control - no antibody



Anti- α_v antibody



Anti- β_1 antibody

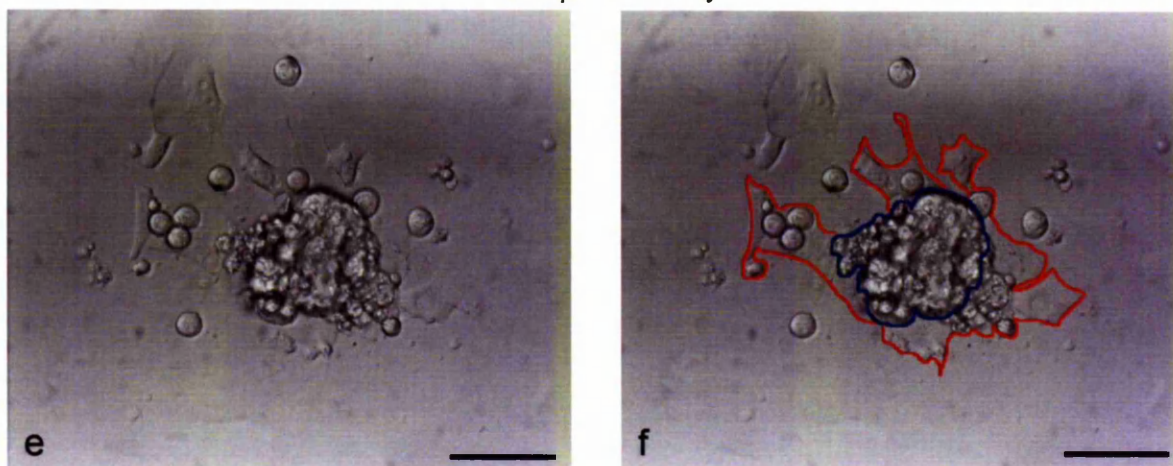
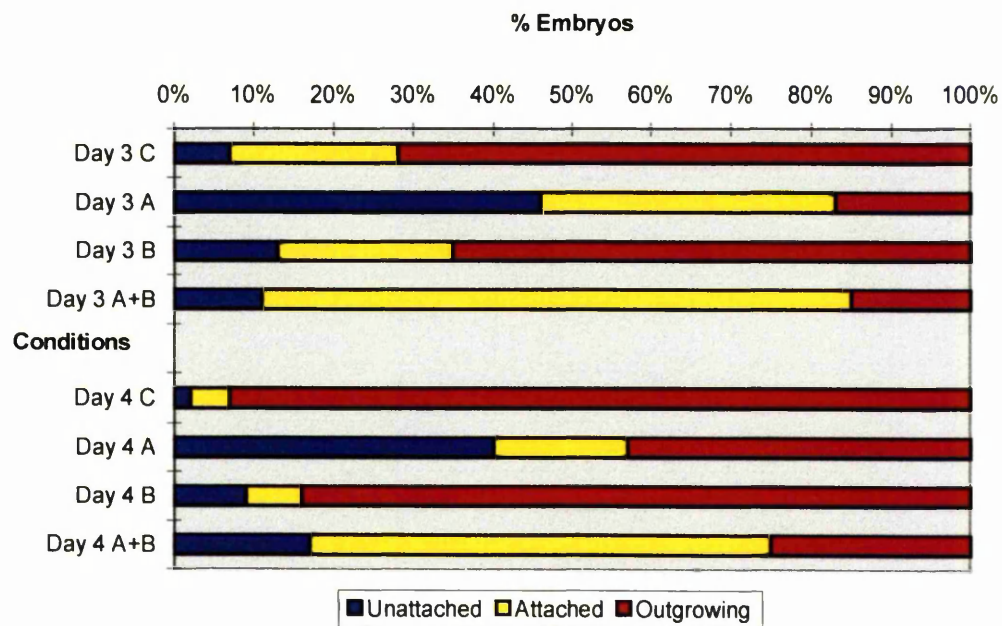
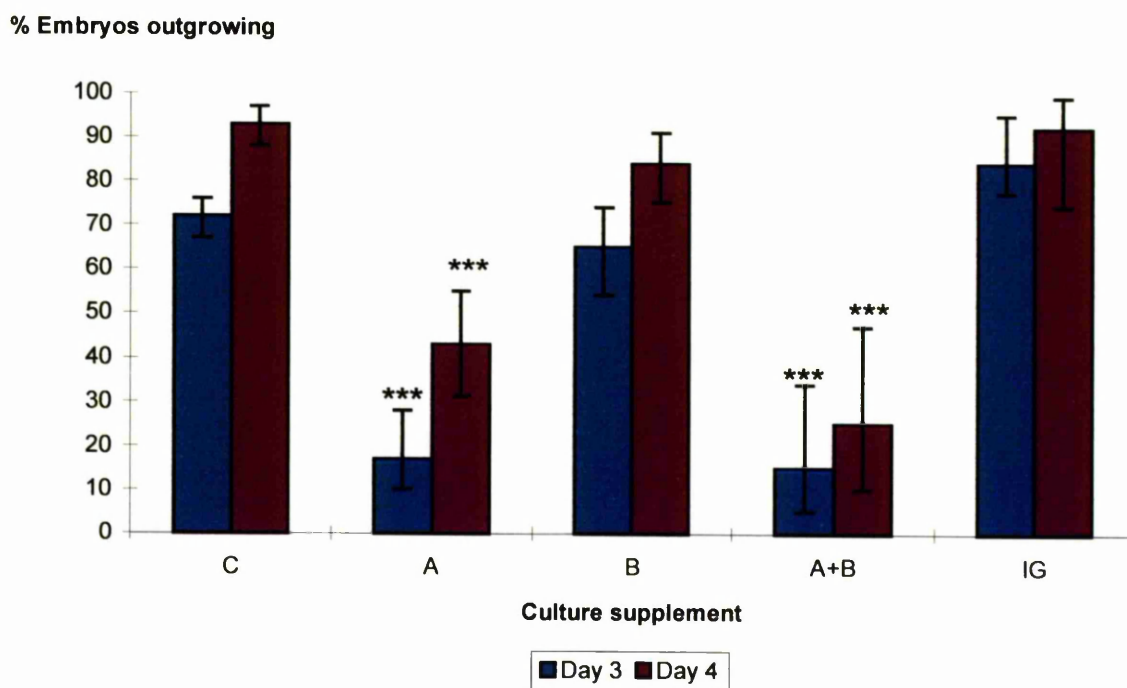


Figure 5.5 Trophoblast outgrowth on vitronectin without serum

Blastocysts cultured on specific substrates in supplemented serum-free medium form trophoblast outgrowths. Outgrowth on vitronectin is blocked by an antibody to α_v family integrins (5.2.7.1). Phase contrast images (a, c, e) are shown in (b, d, f) with the main embryo mass (blue) and outgrowing trophoblasts (red) outlined, scale bar = 100 μ m

Figure 5.6 Trophoblast outgrowth development on vitronectin

[C, control; A, anti- α v; B, anti- β 1]

Figure 5.7 Effect of integrin inhibition on outgrowth on vitronectin

See Table 5.2 for statistical analysis

***Significantly different to control ($p \leq 0.001$)

[C, control; A, anti- αv ; B, anti- $\beta 1$; IG, rat IgG]

Table 5.2 Outgrowth on vitronectin - significance of culture condition

| Comparison of outgrowth success | χ^2 test p value | Level of significance |
|---|---|----------------------------------|
| day 3 control vs. day 3 anti- α v | ≤ 0.0001 | *** |
| day 3 control vs. day 3 anti- β 1 | $= 0.1985$ | NS |
| day 3 control vs. day 3 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 3 control vs. day 3 rat IgG | $= 0.1864$ | NS |
| day 3 anti- α v vs. day 3 anti- β 1 | ≤ 0.0001 | *** |
| day 3 anti- α v vs. day 3 anti- α v+anti- β 1 | $= 1.0$ | NS |
| day 3 anti- β 1 vs. day 3 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 3 anti- α v vs. day 3 rat IgG | ≤ 0.0001 | *** |
| day 3 control vs. day 4 control | ≤ 0.0001 | *** |
| day 3 anti- α v vs. day 4 anti- α v | $= 0.0015$ | ** |
| day 4 control vs. day 4 anti- α v | ≤ 0.0001 | *** |
| day 4 control vs. day 4 anti- β 1 | $= 0.0342$ | NS |
| day 4 control vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 control vs. day 4 rat IgG | $= 0.9729$ | NS |
| day 4 anti- α v vs. day 4 anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- α v vs. day 4 anti- α v+anti- β 1 | $= 0.1903$ | NS |
| day 4 anti- β 1 vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- α v vs. day 4 rat IgG | ≤ 0.0001 | *** |
| day 3 anti- β 1 vs. day 4 anti- β 1 | ≤ 0.0048 | ** |
| day 3 anti- α v+anti- β 1 vs. day 4 anti- α v+anti- β 1 | $= 0.5747$ | NS |

[NS, non-significant; ***, $p \leq 0.001$; **, $p \leq 0.005$]

5.2.7.2 Fibronectin substrate

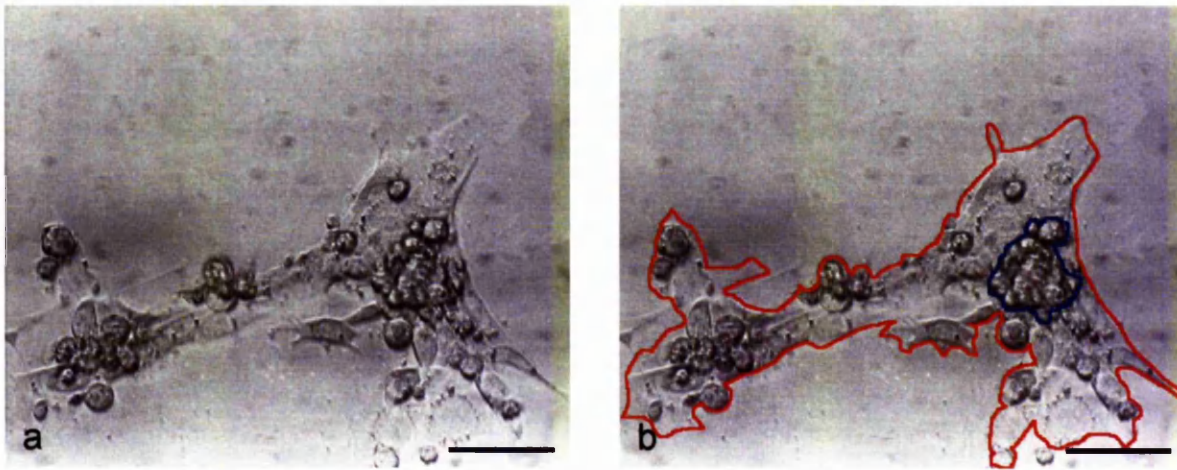
A FN substrate readily supported embryo outgrowth (5.2.4.1). An extensive spread of trophoblast cells was seen radiating from embryos by day 4 (Figure 5.1, 5.2, 5.8a/b).

When anti- αv was added to cultures, little or no outgrowth was seen (Figure 5.8c/d). The proportion of embryos outgrowing was greatly reduced (Figure 5.9) on day 3 (27% versus 58% in controls) and day 4 (15% versus 92% in controls). Outgrowth success was significantly lower upon addition of anti- αv to cultures, compared to controls or when anti- $\beta 1$ was added (Figure 5.10, Table 5.3). No significant increase in the proportion of embryos outgrowing was seen from day 3 to day 4 of culture with anti- αv (Figure 5.10, Table 5.3). Anti- αv had blocked outgrowth by day 3 and that embryos could not overcome this.

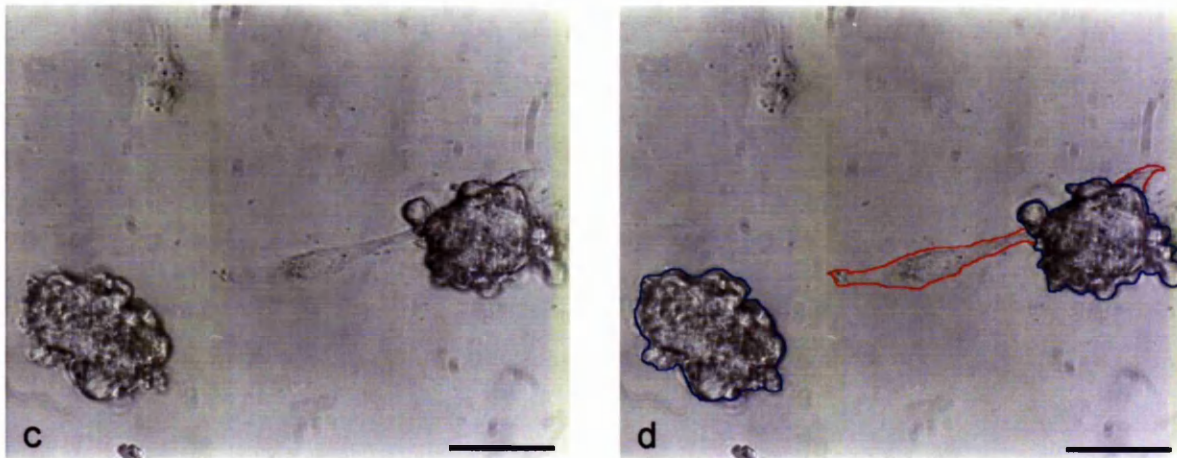
In the presence of anti- $\beta 1$ no difference in outgrowth morphology from controls was apparent (Figure 5.8e/f). The proportions of embryos at each stage of outgrowth were similar with anti- $\beta 1$ compared to controls (Figure 5.9). Anti- $\beta 1$ permitted a significant increase in outgrowth success from day 3 to day 4. However, anti- $\beta 1$ appeared to limit the progression of outgrowth when compared to controls (Figure 5.10, Table 5.3).

No differences were observed between cultures with anti- αv alone or when in combination with anti- $\beta 1$ (Figure 5.9). No significant increase in proportions of embryos outgrowing was seen from day 3 to day 4 when anti- αv was used in combination with anti- $\beta 1$ (12% on day 3, 2% on day 4). αv integrins were an absolute requirement for FN-mediated outgrowth (Figure 5.10, Table 5.3).

Control - no antibody



Anti- α_v antibody



Anti- β_1 antibody

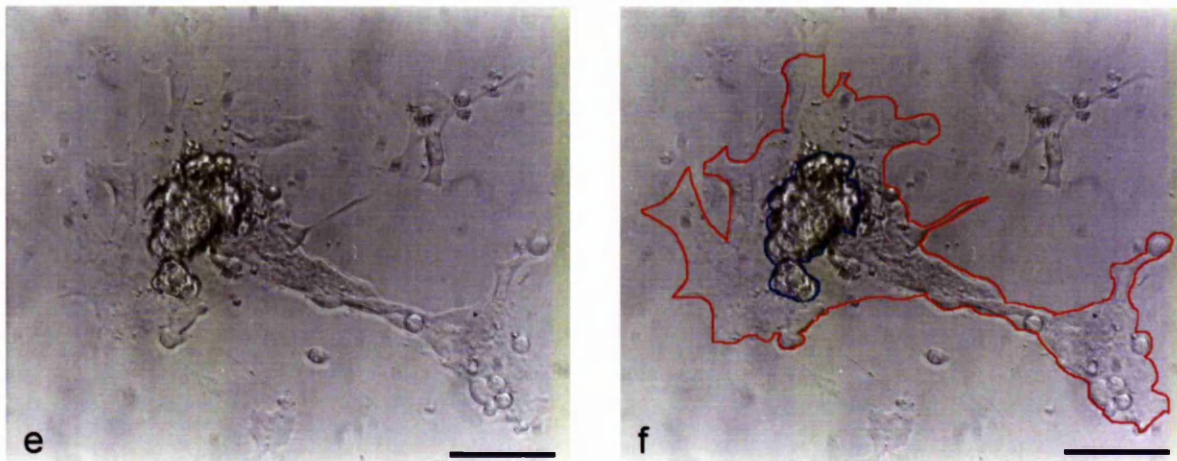
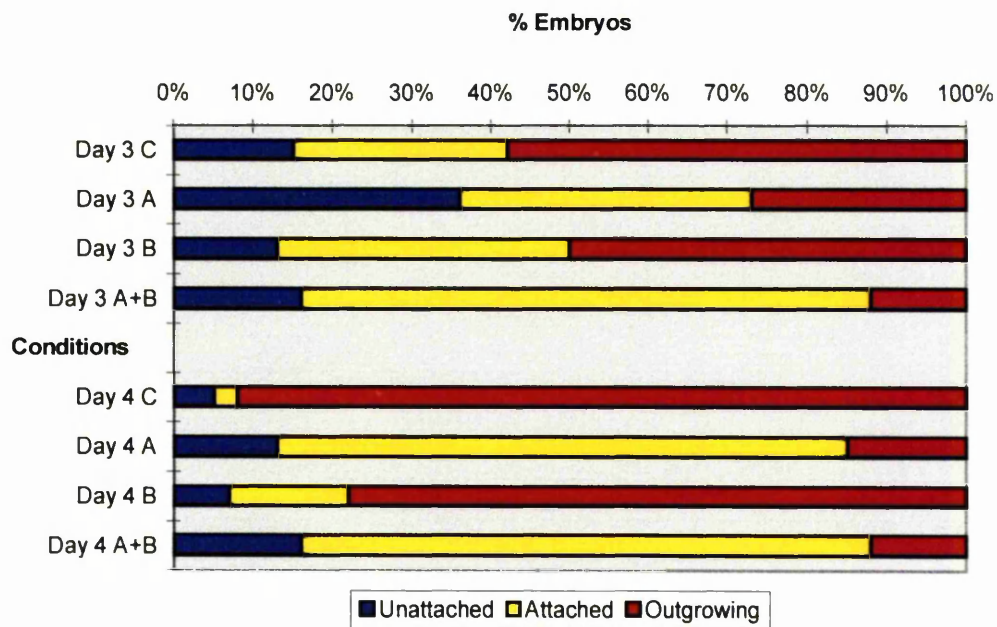
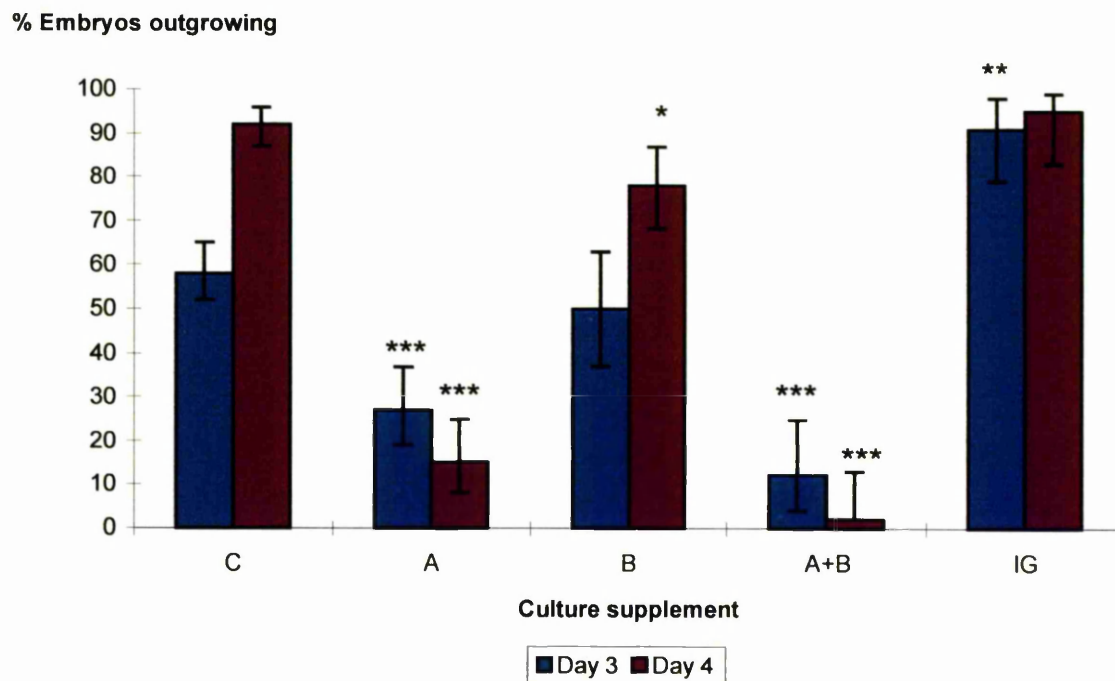


Figure 5.8 Trophoblast outgrowth on fibronectin without serum

Blastocysts cultured on specific substrates in supplemented serum-free medium form trophoblast outgrowths. Outgrowth on fibronectin is blocked by an antibody to α_v family integrins (5.2.7.2). Phase contrast images (a, c, e) are shown in (b, d, f) with the main embryo mass (blue) and outgrowing trophoblasts (red) outlined, scale bar = 100 μ m

Figure 5.9 Trophoblast outgrowth development on fibronectin

[C, control; A, anti- α v; B, anti- β 1]

Figure 5.10 Effect of integrin inhibition on outgrowth on fibronectin

See Table 5.3 for statistical analysis

*** Significantly different to control ($p \leq 0.001$)

** Significantly different to control ($p \leq 0.005$)

* Significantly different to control ($p \leq 0.01$)

[C, control; A, anti- α_v ; B, anti- β_1 ; IG, rat IgG]

Table 5.3 Outgrowth on fibronectin - significance of culture condition

| Comparison of outgrowth success | χ^2 test p value | Level of significance |
|---|---|----------------------------------|
| day 3 control vs. day 3 anti- α v | ≤ 0.0001 | *** |
| day 3 control vs. day 3 anti- β 1 | $= 0.3077$ | NS |
| day 3 control vs. day 3 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 3 control vs. day 3 rat IgG | $= 0.0029$ | ** |
| day 3 anti- α v vs. day 3 anti- β 1 | $= 0.0046$ | ** |
| day 3 anti- α v vs. day 3 anti- α v+anti- β 1 | $= 0.0711$ | NS |
| day 3 anti- β 1 vs. day 3 anti- α v+anti- β 1 | $= 0.0001$ | *** |
| day 3 anti- α v vs. day 3 rat IgG | ≤ 0.0001 | *** |
| day 3 control vs. day 4 control | ≤ 0.0001 | *** |
| day 3 anti- α v vs. day 4 anti- α v | $= 0.0931$ | NS |
| day 4 control vs. day 4 anti- α v | ≤ 0.0001 | *** |
| day 4 control vs. day 4 anti- β 1 | $= 0.0052$ | * |
| day 4 control vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 control vs. day 4 rat IgG | $= 0.0271$ | NS |
| day 4 anti- α v vs. day 4 anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- α v vs. day 4 anti- α v+anti- β 1 | $= 0.0655$ | NS |
| day 4 anti- β 1 vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- α v vs. day 4 rat IgG | ≤ 0.0001 | *** |
| day 3 anti- β 1 vs. day 4 anti- β 1 | ≤ 0.0008 | *** |
| day 3 anti- α v+anti- β 1 vs. day 4 anti- α v+anti- β 1 | $= 0.2260$ | NS |

[NS, non-significant; ***, $p \leq 0.001$; **, $p \leq 0.005$; *, $p \leq 0.01$]

5.2.7.3 EHS matrix substrate

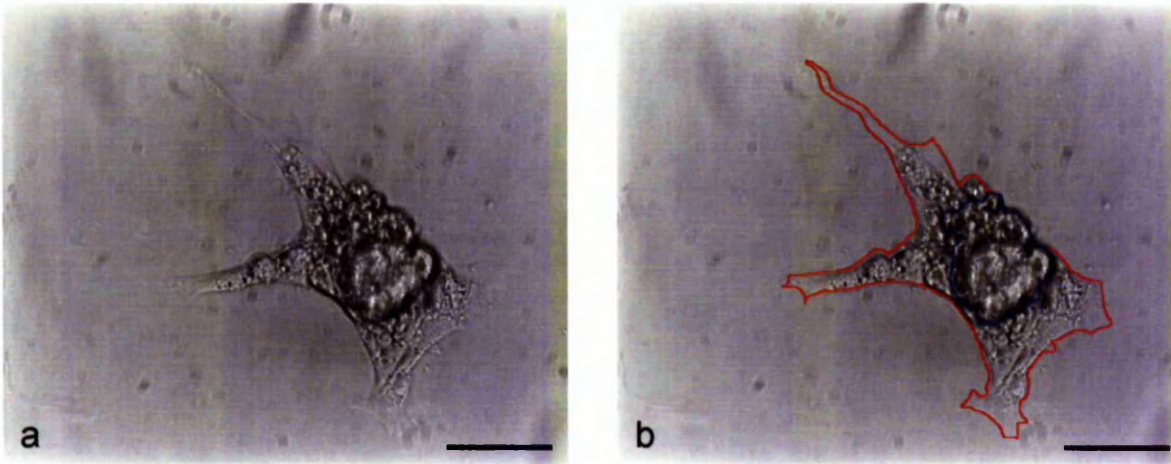
By day 4, blastocysts on EHS matrix demonstrated an ability to outgrow (5.2.4.1), with standard morphology - a lateral spread of trophoblasts radiating away from a flat inner cell mass (Figure 5.11a/b).

Embryos cultured with anti- αv appeared identical to controls (Figure 5.11c/d). Equal proportions of embryos were seen at each stage of outgrowth with anti- αv compared to controls, on days 3 and 4 of outgrowth (Figure 5.12). No significant difference was seen in outgrowth success in the presence of anti- αv compared to controls at either day 3 or 4 (Figure 5.13, Table 5.4).

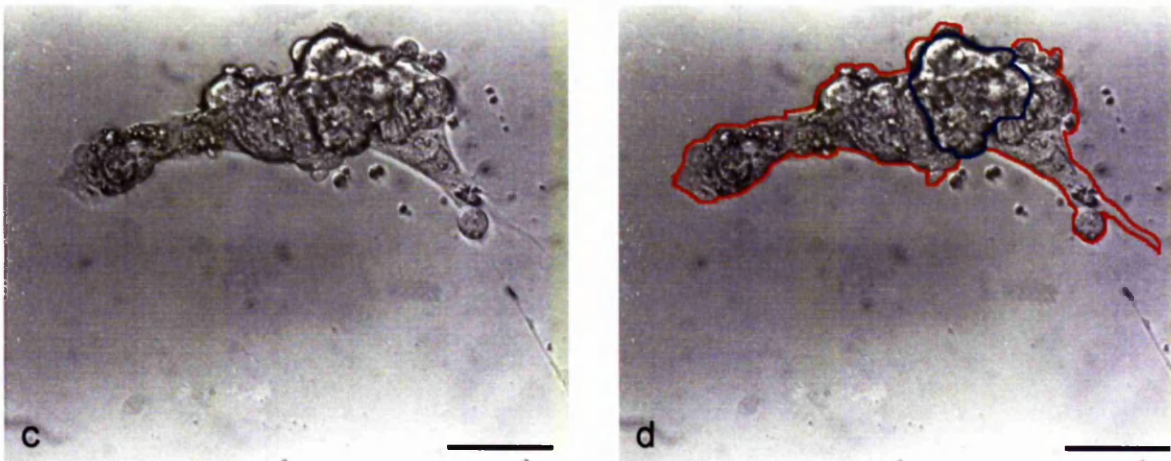
Embryos cultured in the presence of anti- $\beta 1$ showed no signs of outgrowth and were frequently unattached (Figure 5.11e/f). There was no change in the proportion of embryos which remained unattached from day 3 to day 4 (Figure 5.12). The presence of anti- $\beta 1$ prevented any outgrowth on EHS matrix, and was therefore significantly different to cultures supplemented with anti- αv or no antibody (Figure 5.13, Table 5.4). As no outgrowths ('successes') were seen with anti- $\beta 1$ it was not possible to calculate p values using chi-squared test for this group (Table 5.4). $\beta 1$ integrins were an absolute requirement for outgrowth on EHS matrix and inhibition of $\beta 1$ function was not overcome by day 4 of culture (Figure 5.13).

Embryos cultured in the presence of anti- $\beta 1$ in conjunction with anti- αv showed no outgrowth, as was seen with anti- $\beta 1$ alone (Figure 5.12). There was no difference between the extent of outgrowth inhibition between embryos with anti- $\beta 1$ alone, or in combination with anti- αv , at either day 3 or 4 (Figure 5.13, Table 5.4).

Control - no antibody



Anti- α_v antibody



Anti- β_1 antibody

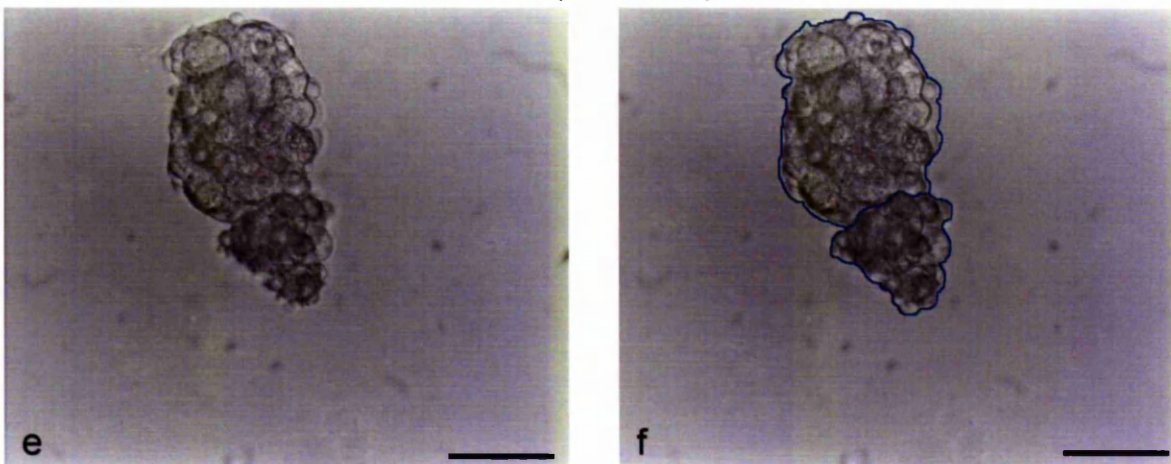
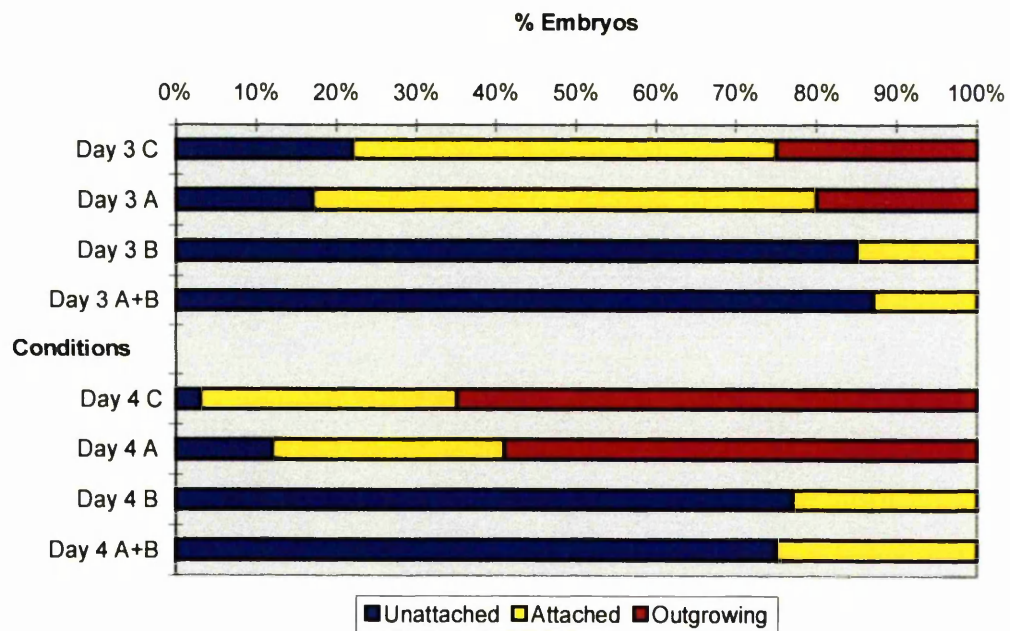
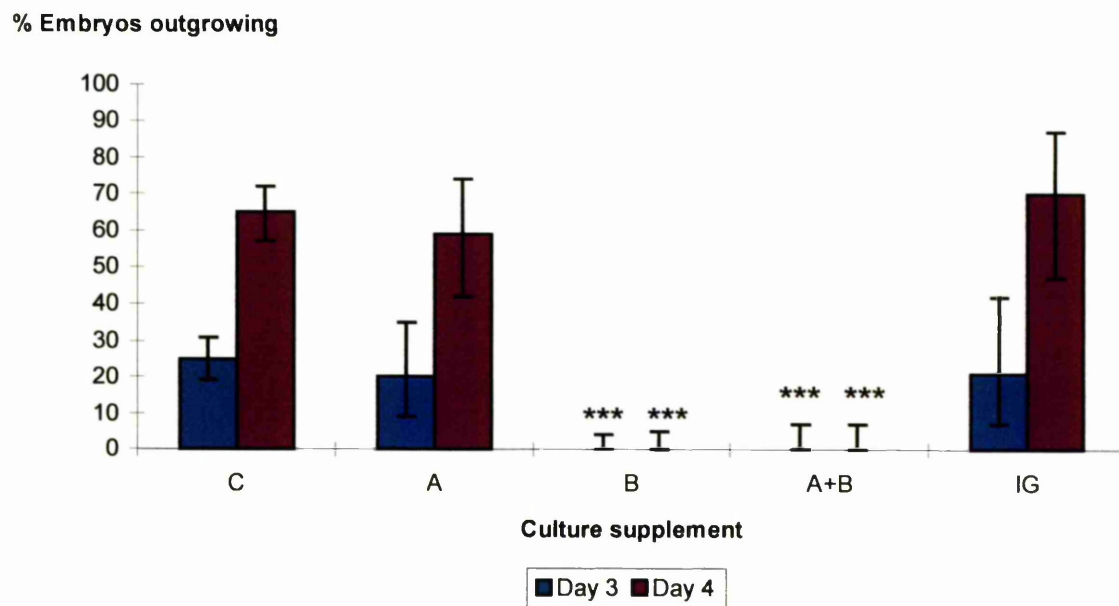


Figure 5.11 Trophoblast outgrowth on EHS matrix in the absence of serum

Blastocysts cultured on specific substrates in supplemented serum-free medium form trophoblast outgrowths. Outgrowth on EHS matrix is blocked by an antibody to β_1 family integrins (5.2.7.3). Phase contrast images (a, c, e) are shown in (b, d, f) with the main embryo mass (blue) and outgrowing trophoblasts (red) outlined, scale bar = 100 μ m

Figure 5.12 Trophoblast outgrowth development on EHS matrix

[C, control; A, anti- α v; B, anti- β 1]

Figure 5.13 Effect of integrin inhibition on outgrowth on EHS matrix

See Table 5.4 for statistical analysis

***Significantly different to control ($p \leq 0.001$)

[C, control; A, anti- αv ; B, anti- $\beta 1$; IG, rat IgG]

Table 5.4 Outgrowth on EHS matrix - significance of culture condition

| Comparison of outgrowth success | χ^2 test p value | Level of significance |
|---|---|----------------------------------|
| day 3 control vs. day 3 anti- α v | = 0.6157 | NS |
| day 3 control vs. day 3 anti- β 1 | ≤ 0.0001 | *** |
| day 3 control vs. day 3 anti- α v+anti- β 1 | = 0.0003 | *** |
| day 3 control vs. day 3 rat IgG | = 0.8757 | NS |
| day 3 anti- α v vs. day 3 anti- β 1 | = 0.0002 | *** |
| day 3 anti- α v vs. day 3 anti- α v+anti- β 1 | = 0.0046 | ** |
| day 3 anti- β 1 vs. day 3 anti- α v+anti- β 1 | E | NS |
| day 3 anti- α v vs. day 3 rat IgG | = 0.8471 | NS |
| day 3 control vs. day 4 control | ≤ 0.0001 | *** |
| day 3 anti- α v vs. day 4 anti- α v | = 0.0007 | *** |
| day 4 control vs. day 4 anti- α v | = 0.5684 | NS |
| day 4 control vs. day 4 anti- β 1 | ≤ 0.0001 | *** |
| day 4 control vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 control vs. day 4 rat IgG | = 0.8324 | NS |
| day 4 anti- α v vs. day 4 anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- α v vs. day 4 anti- α v+anti- β 1 | ≤ 0.0001 | *** |
| day 4 anti- β 1 vs. day 4 anti- α v+anti- β 1 | E | NS |
| day 4 anti- α v vs. day 4 rat IgG | = 0.5449 | NS |
| day 3 anti- β 1 vs. day 4 anti- β 1 | E | NS |
| day 3 anti- α v+anti- β 1 vs. day 4 anti- α v+anti- β 1 | E | NS |

[NS, non-significant; E, no successes; ***, $p \leq 0.001$; **, $p \leq 0.005$]

Part 2 - Investigation of integrin function in vivo: feasibility studies

5.2.8 Setting up and assessment of the intra-uterine injection method

Initial experiments in mice allowed an estimate of normal implantation rates to be made and also development of a standard intra-uterine injection technique in preparation for integrin inhibition experiments.

Four groups containing ten mice in total (all demonstrating a vaginal plug and therefore considered pregnant) underwent unilateral intra-uterine injection with 20µl of sterile saline (2.1.12). Injections were made 86.5 to 89.5 hours post-mating (day 4 of pregnancy; Zhu *et al.*, 1995) under general anaesthetic. The uterine horn and associated structures of the uninjected side were not manipulated and the peritoneum remained intact on that side. On either day 6 or day 7 of pregnancy the animals were killed, the uterine horns and associated structure were removed and examined under X10 magnification with overhead lighting. Three of the mice showed no corpora lutea or implantation sites and were therefore considered non-pregnant. The numbers of corpora lutea and implantation sites for the remaining seven animals were counted on both sides and the implantation rate (Table 5.5) was calculated as:

$$\text{Implantation rate} = (\text{implantation sites} \div \text{corpora lutea}) \times 100$$

The results demonstrated that intra-uterine injection of saline (Table 5), reduces the implantation rate to one third of that seen in the uninjected uterine horns. By this stage in the investigation the technique was well practiced, it was therefore still considered worthy of a further small study using function-blocking antibodies to integrins.

5.2.8.1 Intra-uterine injection of a function blocking anti- α v antibody

Three groups of mice (eleven animals in total) underwent bilateral intra-uterine injection 86.5 to 89.5 hours post-mating. One horn was injected with 20 μ l of affinity purified anti-mouse α v integrin antibody (rat IgG at 500 μ g/ml) and the other horn was injected with 20 μ l of affinity purified rat IgG (500 μ g/ml). The substances to be injected were labeled by an independent investigator and decoded only once the corpora lutea and implantation sites had been counted, on day 6 or 7 of pregnancy. One of the animals was found not to be pregnant; the implantation rates for the remaining ten mice are shown on Table 5.5.

It was clear that there were no significant differences in implantation rate between the horns injected with anti- α v antibody or control IgG. In addition the implantation rate of both groups was only half that of the uninjected uterine horns in the previous study. This investigation highlighted the disruptive nature of such in vivo experiments and was not extended.

Table 5.5 Effect of intra-uterine injections on implantation rate

| Injection type (number animals) | Corpora lutea (CL) | Implantation sites (IS) | Implantation rate (IS÷CL) x 100 |
|------------------------------------|-----------------------|----------------------------|------------------------------------|
| Uninjected (n=7) | 41 | 36 | 88 |
| 20µl saline (n=7) | 33 | 9 | 27 |
| 10µg rat IgG (n=10) | 62 | 26 | 42 |
| 10µg anti-αv (n=10) | 67 | 34 | 51 |

5.3 DISCUSSION

It was shown in Chapter 4 that integrins of the αv and $\beta 1$ families are expressed by human and mouse blastocysts, and that these were localised in distinct staining patterns in mouse trophoblasts outgrowing in the presence of serum (Chapter 4). To further this work the receptors involved in trophoblast outgrowth were examined. Blastocysts were cultured in the absence of serum, on defined substrates. The substrates used in this study were chosen on the basis of their potential as ligands for either αv or $\beta 1$ integrins, and their occurrence in the mouse and human endometrial ECM. BSA was used as a control substrate (Armant *et al.*, 1986a), on which some embryo attachment but little outgrowth was observed.

The role of αv family integrins in trophoblast outgrowth was examined using FN, VN and TSP substrates. TSP did not support embryo attachment or outgrowth. Conformation of adsorbed TSP is profoundly affected by calcium, and cell attachment to TSP is calcium dependent (Lawler *et al.*, 1988). Nevertheless, whether TSP adsorption buffer was prepared with addition or omission of calcium, the TSP coating did not promote embryo attachment or outgrowth in the present study. A previous study has described trophoblast outgrowth on TSP, although the role of calcium was not explored (O'Shea *et al.*, 1990); however on examination of the micrographs of embryos outgrowing on TSP, the 'outgrowths' appear to be flattened blastocysts with little evidence of spreading or migratory trophoblasts. These cultures do not resemble successful outgrowths seen either in my work or that of others (Armant *et al.*, 1986b; Carson *et al.*, 1988; Sutherland *et al.*, 1988), on other substrates at the equivalent stage in development.

On day 3, the proportion of embryos outgrowing in serum-containing medium, which provides a VN substrate (Hayman *et al.*, 1985), is greater than that for embryos cultured on VN (or other substrates) in the absence of serum. This is probably due to additional nutrients and growth and/or attachment promoting factors present in serum. It appeared that these factors accelerated outgrowth

development; as by day 4, comparable (maximum) numbers of embryos are outgrowing in the presence of serum (Figure 4.5d) or in its absence on VN (Figure 5.5a). Control embryos not outgrowing on this substrate by day 4 ($\leq 8\%$) were probably abnormal. The proportion of embryos which were abnormal in outgrowth experiments was similar to the implantation failure rate in vivo (12%), in control uninjected uterine horns (5.2.8) as indicated in Table 5.5.

α_v integrins were required for outgrowth on VN. By day 4 on VN it seemed that attached embryos partially overcome outgrowth inhibition by anti- α_v . This effect was independent of β_1 integrin function as at this stage; the proportion of embryos outgrowing in the presence of both anti- α_v and anti- β_1 is significantly more than that seen on a BSA substrate ($p = 0.0051$). This suggested that an alternative mechanism for trophoblast outgrowth on VN became important. Another receptor for VN is $\alpha_{IIb}\beta_3$, an integrin that recognises RGD. Trophoblast outgrowth on VN appears RGD dependent (Armant *et al.*, 1986b) and $\alpha_{IIb}\beta_3$ is expressed by trophoblasts (Yelian *et al.*, 1995). Therefore, $\alpha_{IIb}\beta_3$ may compensate for the inhibition of α_v function. In this case, trophoblast outgrowth in the presence of anti- α_v may be delayed until cells upregulate α_{IIb} and thus produce a new partner for β_3 . This possibility could be examined using a function blocking antibody to α_{IIb} and/or β_3 , either alone or in combination with anti- α_v or anti- β_1 . Alternatively, non-integrin dependent interactions with VN may become important in the presence of anti- α_v . The most likely mechanism for this would be interaction between trophoblast HSPG (Carson *et al.*, 1993) and the heparin binding region of VN. Previous work has suggested that trophoblast outgrowth on other substrates (FN or LM) involves heparin (Farach *et al.*, 1987). Another explanation for trophoblasts overcoming the inhibition of α_v function is that α_v subunit expression is upregulated, producing sufficient free α_v integrins to support trophoblast outgrowth; however this is less likely as the antibody is present in excess.

Outgrowth of trophoblast from embryos on FN was inhibited by anti- α_v , and partial inhibition of outgrowth in the presence of anti- β_1 was evident by day 4 on

FN. In contrast to VN, embryos on FN cannot overcome inhibition of α_v function, suggesting that alternative FN receptors (e.g. $\alpha_{IIb}\beta_3$) are less important on this substrate. In support of this is the observation that addition of both anti- α_v and anti- β_1 to cultures reduces outgrowth to a level not significantly different to that seen on a BSA substrate. It has been reported that on exposure to immobilised FN, blastocysts upregulate both β_1 and β_3 integrins (Schultz & Armant, 1995); however it was unlikely that they could be upregulated to such an extent as to overcome outgrowth inhibition by an excess of antibody in the present study. Trophoblast cells outgrowing on FN have been shown to express α_5 , α_v , α_{IIb} , β_1 and β_3 integrin subunits, all of which localise to focal adhesions. Addition of either anti- β_1 or anti- β_3 to these cultures significantly reduced outgrowth area, but the proportion of embryos outgrowing was not described (Yelian *et al.*, 1995). It has been suggested that trophoblast outgrowth on FN is mediated by heparin (Farach *et al.*, 1987). The functional studies I have carried out and those published by others (Yelian *et al.*, 1995; Schultz & Armant, 1995) indicate that integrin interactions dominate over those involving heparin, for trophoblast outgrowth on FN.

The integrin $\alpha_v\beta_3$ can bind TSP, VN and FN, recognising the RGD amino acid recognition site in each of these ECM components (Sugimori *et al.*, 1997). Therefore the apparent differences between the ability of these ligands to support trophoblast outgrowth suggests a mechanism more complex than simply integrin-RGD binding. One mechanism which may regulate the ligand binding of $\alpha_v\beta_3$ is the relative contribution of other α_v family integrins.

All α_v family substrates used in the present study (TSP, VN, FN) are present in the extracellular matrix of the uterine stroma, and could be encountered by trophoblast cells during the invasion stages of implantation in utero. It was surprising that TSP did not support outgrowth, as this substrate is regulated by progesterone in the endometrium (Iruela-Arispe *et al.*, 1996; Corless *et al.*, 1992) and is expressed at the trophectoderm surface (O'Shea *et al.*, 1990). It was expected that VN would support trophoblast outgrowth, as this substrate is a

ligand for many α_v family integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$), although its expression is limited in the uterine stroma (Seiffert *et al.*, 1994; Aplin *et al.*, 1988). FN served as a positive control as its ability to support trophoblast outgrowth has been reported previously (Armant *et al.*, 1986a). FN is a ligand for several α_v and β_1 family integrins and is a major component of the endometrial stromal ECM (Babiarz *et al.*, 1996). OST and TN are also α_v family integrin ligands, to my knowledge these have not been used as substrates for embryo outgrowth. OST, a ligand for several α_v -containing integrins, is upregulated around the time of implantation at the uterine epithelium (Brown *et al.*, 1992) and thus may be important in blastocyst attachment. TN expression increases in the mouse uterine stroma, specifically at the site of blastocyst attachment (Julian *et al.*, 1994) and may be involved in trophoblast invasion. Both proteins may therefore play an important role in implantation and are worthy of further study.

Addition of anti- β_1 to embryos cultured on EHS matrix produced complete inhibition of embryo outgrowth, with most embryos remaining unattached. This is perhaps not surprising as the two components of EHS matrix, LM-1 and COL IV, are both ligands for several β_1 family integrins. Mouse LM-1 does contain an RGD site which could potentially serve as a ligand for $\alpha_v\beta_3$, but this site is cryptic on LM-1 (Armant, 1991). As with FN, trophoblast outgrowth on LM has been reported to involve heparin (Farach *et al.*, 1987). This however was not evident in my own or other work (Armant, 1991).

Although the proportion of embryos outgrowing on VN is not affected by addition of anti- β_1 , this antibody did have an effect on the morphology of some trophoblasts. These cells, although migratory, appeared unable to spread, and remained rounded. This phenomenon was seen in most anti- β_1 containing cultures on VN but only a minority of the trophoblast cell population was affected. One aspect of embryo outgrowth on VN which is difficult to explain is the proportion of unattached embryos seen with various culture conditions. It is perhaps not surprising that with anti- α_v there are many more embryos unattached than with anti- β_1 . However when both antibodies are present, few

embryos are unattached. It seems that addition of anti- $\beta 1$ to cultures promotes embryo attachment, independent of any effect on embryo outgrowth. A possible explanation for this is that integrin ligation by antibodies causes upregulation of receptors which can mediate embryo attachment but not outgrowth.

EHS matrix provides a substrate that is similar to the uterine epithelial basement membrane, its major components are LM and COL IV (Farrar & Carson, 1992; Aplin *et al.*, 1988), both ligands for $\beta 1$ family integrins. It is not clear why EHS matrix (containing LM-1 and COL IV) was not as efficient at supporting trophoblast outgrowth as VN or FN. The observation that embryo attachment and/or outgrowth on EHS matrix may be delayed by 24 hours compared to VN or FN indicates that embryos lacked certain components required for interaction with EHS and that these were subsequently acquired, allowing outgrowth to proceed. Upregulation of integrins upon ligand exposure has previously been demonstrated in blastocysts; where soluble FN led to upregulation of $\beta 1$ and $\beta 3$ integrins (Schultz & Armant, 1995). The retarded development of outgrowths on EHS matrix in the present study differs from previous studies which have shown that LM-1 (alone) supports outgrowth in a manner comparable to FN (Armant *et al.*, 1986a). There is conflicting evidence regarding the ability of COL IV to support outgrowth (Carson *et al.*, 1988; Armant *et al.*, 1986a). Outgrowth on a mixed substrate such as EHS matrix may be more complex than on a single substrate, involving multiple signalling pathways. In addition to LM-1 and COL IV, EHS matrix also contains other minor ECM components. It would be of interest to determine which components of EHS matrix are used as ligands by outgrowing trophoblasts. Trophoblast invasion is controlled by a range of growth factors some of which have inhibitory action. For example, TGF- β has been suggested to inhibit trophoblast invasion (Giudice & Saleh, 1995). In addition to ECM components, inhibitory factors may also be present in EHS matrix which limit trophoblast cell attachment, spreading or migration.

While EHS matrix contains LM-1, there are several additional LM isoforms present in human endometrium, including LM-2 and LM-4 which show hormonal

regulation (Church *et al.*, 1997, 1996). The ability of these to support trophoblast outgrowth has not yet been investigated. A mixture of LM-2 and LM-4 can support adhesion of BeWo choriocarcinoma-derived cells (John Aplin, personal communication).

The data collected in the present study, and that of others (Armant *et al.*, 1986b; Farach *et al.*, 1987; Sutherland *et al.*, 1993; Schultz & Armant, 1995; Yelian *et al.*, 1995) suggests that trophoblast invasion is a complex process involving cross-talk between α_v and β_1 family integrins, and other adhesion systems. Embryos lacking β_1 integrins through gene knockout can invade the uterine basement membrane, but die shortly after (Fassler & Meyer, 1995; Stephens *et al.*, 1995). As the uterine epithelial basement membrane is largely composed of β_1 family integrin ligands (LM, COL) and HSPG, it is likely that heparin-mediated interaction are important at this stage. This is supported by the observation that COL (types I, III or IV) may not support trophoblast outgrowth (Armant *et al.*, 1986a). Although studies by Carson *et al.* (1988) suggest that several COL types (I to VI) can support outgrowth but with differing profiles of outgrowth development. Once an embryo has breached the basement membrane it encounters an ECM containing LM, COL and FN. Mice lacking the gene encoding α_v do not die until birth (Fassler *et al.*, 1996; Hynes, 1996). This is surprising considering the contribution that α_v family ligands make to the uterine extracellular environment. In the present study α_v was shown to be crucial for trophoblast interaction with FN, while β_1 integrins (e.g. $\alpha_5\beta_1$) were not required. In a study using cells from α_5 deficient mice, cells required α_v function for adhesion to FN (otherwise mediated by $\alpha_5\beta_1$ or $\alpha_4\beta_1$). These knock out cells did not upregulate α_v compared to wild type, but α_v was recruited to focal adhesions. Therefore in this instance α_v could functionally compensate for lack of $\alpha_5\beta_1$ (Yang & Hynes, 1996); but in the present study α_v was an absolute requirement. While integrin knockout experiments provide information regarding integrin requirement, they do not allow studies of specific cellular interactions. Conversely, in vitro experiments using inhibitory antibodies, such as the model of implantation used in the present study, can provide detailed information

regarding specific molecular interactions during cellular processes such as trophoblast outgrowth.

Ultimately it is likely that both integrin and heparin mediated adhesion systems are important in the complex mechanism of implantation in utero, where multiple ligands are available for trophoblast interaction. Providing mixed substrates for trophoblast outgrowth in vitro (Armant & Kameda, 1994) would allow more complex adhesive interactions to be studied. Trophoblast ligand preferences, and any temporal changes in these, could be examined further to determine the relative importance of heparin-mediated interactions and those involving αv or $\beta 1$ family integrins. To identify specific integrins capable of mediating trophoblast outgrowth, anti-integrin antibodies which recognise ligand binding sites could also be used as substrates for embryo outgrowth.

Introduction of substances, such as inhibitory antibodies, into the uterine cavity prior to implantation could be of great value in examining implantation in utero. In the mouse (Swiss-Webster strain), precisely timed intra-uterine injection of an antibody directed against the Le^y carbohydrate epitope has demonstrated a role for this oligosaccharide in implantation (Zhu *et al.*, 1995). Similar experiments have also suggested the necessity for $\alpha v \beta 3$ at implantation (Illera *et al.*, 1997), although the details of this work have yet to be reported. With the potential importance of this experimental technique, it is perhaps surprising that its use has not been described more often.

The necessarily invasive nature of intra-uterine injection inevitably leads to mechanical disturbance of the uterine environment, and disruption of normal implantation. In my hands, introduction of a small volume of fluid into the uterine lumen caused a dramatic reduction in implantation rate, so that any additional effect of inhibitory antibodies were statistically very difficult to demonstrate. In the study of Zhu *et al.* (1995), at 87 or 93 hours postcoitum specific inhibition of implantation by anti-Le^y was demonstrated; the implantation rate was reduced to ~30%, versus ~90% seen with PBS. However at 80 hours post coitum, injection

of either PBS or anti-Le^y reduced the implantation rate to 25% of that of uninjected controls. Outside these times, no significant effect on implantation was seen with either substance (Zhu *et al.*, 1995). It is possible that differences in the exact timing of implantation exist, such that the time of intra-uterine injection needs to be determined empirically for each mouse strain. However, the value of further experiments such as those using anti-integrin antibodies remains questionable.

CHAPTER 6

Production of $\beta 5$ integrin fusion proteins for immunisation

6.1 INTRODUCTION

In vitro outgrowth experiments provided a model for investigating the function of individual integrin subunits in trophoblast outgrowth. The study was restricted by the availability of function blocking antibodies that cross-react with mouse integrin subunits. Therefore the aim of the following work was to provide reagents for further functional studies. Having shown the distinct expression pattern of the $\beta 5$ integrin subunit in mouse endometrium and outgrowths, and as little other published work is available on the expression and function of $\beta 5$ in implantation, this subunit was the focus of this work. Following a collaboration we obtained a cloned cDNA of mouse $\beta 5$ integrin subunit that was then used for fusion protein production. The ultimate aim of producing $\beta 5$ fusion proteins for subsequent immunisation and antibody production required the fusion protein to be as similar to murine $\beta 5$ as possible. As the importance of glycosylation in integrin function is not known, the insect cell baculovirus expression system, in which eukaryotic glycosylation occurs, was the method of choice.

Polymerase chain reaction was used to engineer DNA fragments coding for full length and truncated $\beta 5$ for subcloning into the baculovirus transfer vector pAcSecG2T. The truncated fragment would produce fusion protein consisting of the ectodomain of mouse $\beta 5$, containing the integrin-ligand binding sites and therefore was most likely to provide a function blocking antibody following immunisation. The vector pAcSecG2T used for co-transfection of the $\beta 5$ cDNA into the insect cells was chosen for several important features. This vector uses the strong polyhedrin promoter for transcription of an inserted gene in the late stages of insect cell viral infection. Upstream of the multiple cloning site (MCS) is a glutathione S-transferase (GST) coding region, allowing synthesis of GST-tagged fusion proteins, which can be readily purified using the glutathione affinity system. Furthermore a gp67 signal sequence is included to promote secretion of the fusion protein. The insect baculovirus expression system is summarised in Figure 6.1.

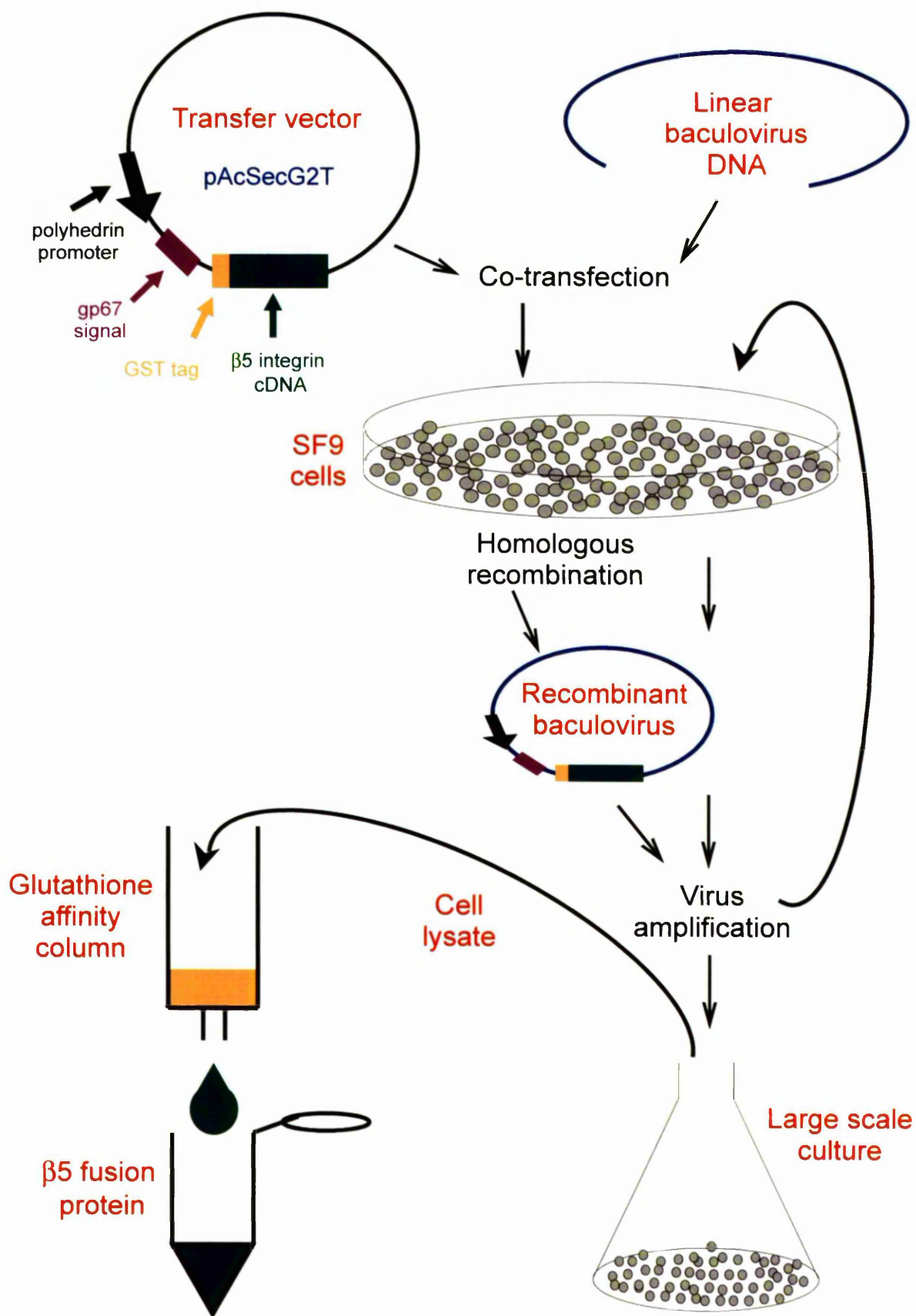


Figure 6.1 Summary of the insect cell baculovirus expression system

6.2 RESULTS

Part 1 - Subcloning of $\beta 5$ DNA fragments into a baculovirus transfer vector

6.2.1 Purification of the mouse $\beta 5$ integrin DNA fragment

The vector pcDNAneo1 (Invitrogen) containing a partially sequenced ~ 2.9 Kb cDNA insert complementary to mouse $\beta 5$ integrin subunit was a generous gift of Scott Baldwin (Wistar Institute, Philadelphia). After amplification of pcDNAneo1- $\beta 5$ transformed into MC1061/p3, purified plasmid DNA was double-digested with Xba I and Xho I to liberate the $\beta 5$ insert. Products were separated (Figure 6.2a) and the band corresponding to the $\beta 5$ insert was excised from the gel and purified.

6.2.2 Restriction enzyme site mapping of mouse $\beta 5$ integrin DNA

A restriction enzyme (RE) profile of the $\beta 5$ insert was required to subclone $\beta 5$ DNA fragments into the baculovirus transfer vector pAcSecG2T within the MCS containing Bam HI, Eco RI and Sma I restriction enzyme sites. The known partial mouse sequence (courtesy of Scott Baldwin; illustrated in Figure 2.2) corresponding to bases 30-278 (MS $\beta 5$ -1), 488-724 (MS $\beta 5$ -2), and 2053-2426 (MS $\beta 5$ -3) of human $\beta 5$ (GenBank accession code: M35011) were analysed using the gcg-Sequence Editor Map program (Human Genome Mapping Project) and the RE maps of the three regions (Appendix 5) were compared. Of the three RE sites within the MCS of pAcSecG2T, only Bam HI did not cut the analysed regions of mouse $\beta 5$. Two of the partial sequences contained a unique RE site that was also present in pAcSecG2T and therefore could potentially be used for orientation of $\beta 5$ fragments ligated into pAcSecG2T. A Hind III digest of $\beta 5$ was predicted to cut at base number 30 of MS $\beta 5$ -2. A Bst XI digestion was expected to cut at base number 148 of MS $\beta 5$ -3. As this enzyme analysis was only of partial $\beta 5$ sequences, the results were confirmed experimentally by digestion of the purified $\beta 5$ insert.

Purified insert (6.2.1) was digested (2.2.5) with Bam HI, Bst XI and Hind III (Figure 6.2a). As anticipated, Bam HI did not cut the $\beta 5$ insert. Bst XI yielded two fragments (~ 650 bp and ~ 2.2 Kb) and Hind III also yielded two fragments (~ 850 bp

and ~2Kb) of the sizes predicted. Alignment of the known mouse $\beta 5$ DNA sequence with the full human $\beta 5$ DNA sequence showed that the Bst XI site was within the predicted transmembrane domain of $\beta 5$ and therefore would not be present in an ectodomain-only fragment of $\beta 5$. Hind III was within the extracellular domain of $\beta 5$ and therefore would be present in an ectodomain-only fragment of $\beta 5$, allowing orientation of either the full length or ectodomain fragment of $\beta 5$ to be determined, once cloned into pAcSecG2T.

6.2.3 PCR amplification of full length and truncated $\beta 5$ DNA fragments for ligation into pAcSecG2T

Three primers were designed (2.2.8) in order to amplify the coding regions of both full length and the ectodomain domain of $\beta 5$ and to add Bam HI RE sites to both ends of the DNA fragments (allowing ligation into pAcSecG2T). The forward primer annealed at bases 7-21 of MS $\beta 5$ -1 (primer EX). The reverse primers were designed to anneal within MS $\beta 5$ -3 at bases 111-128 (primer TM) and 354-368 (primer CY). Each primer had a Bam HI RE site added in-frame at the 5' end. Primer TM had the TCA stop codon inserted in-frame between the Bam HI site and the region complementary to $\beta 5$ (see section 2.2.8). A range of annealing temperatures from 60°C to 70°C were used to optimise both primer pairs for amplification of single products of the appropriate size (2.2.9), corresponding to either $\beta 5$ DNA fragment. The primer pair EX-CY was shown to anneal specifically to $\beta 5$ at 67°C to 68°C yielding a product of ~2.4Kb as predicted (Figure 6.2b). The primer pair EX-TM required an annealing temperature of exactly 67°C to amplify a single product of the expected size ~2.15Kb (Figure 6.2b).

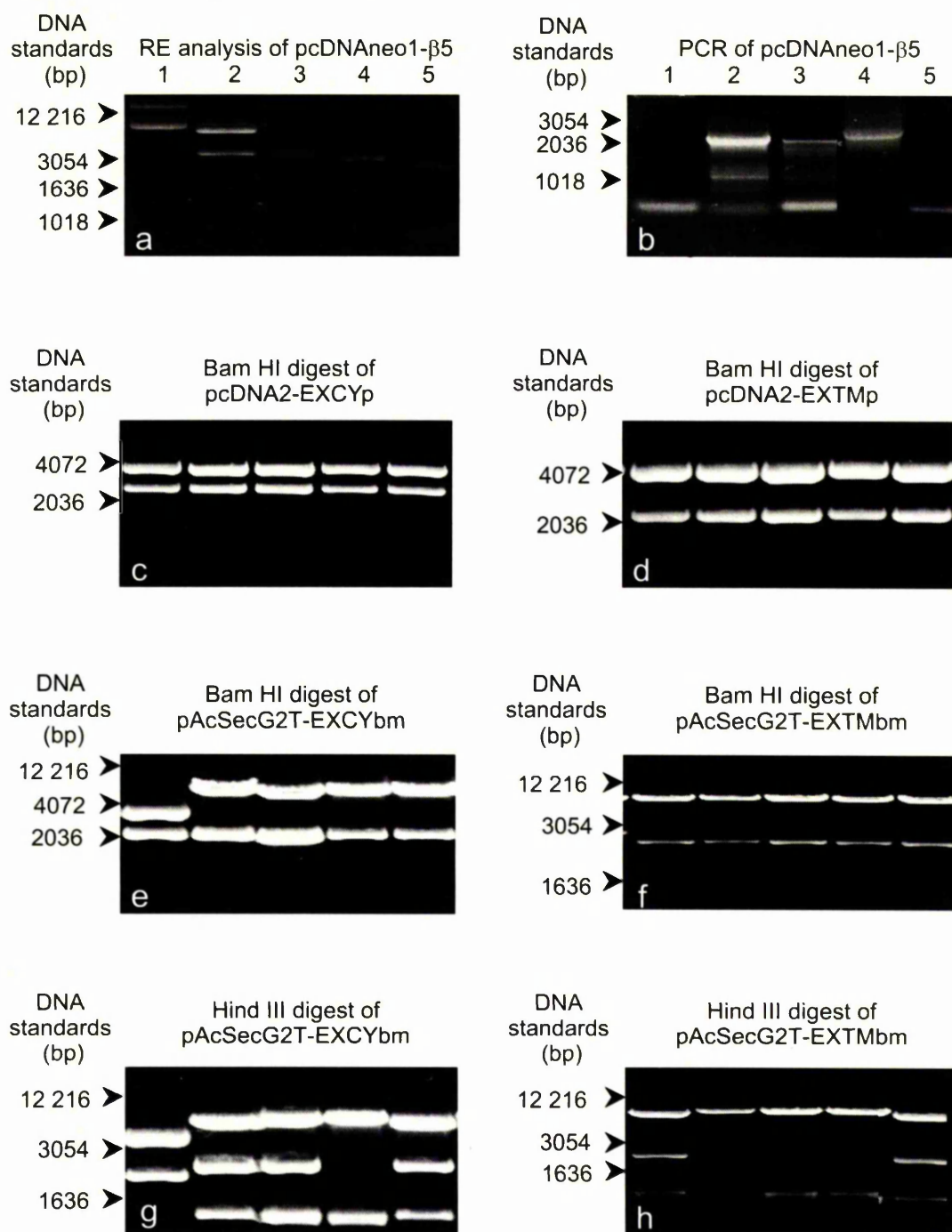


Figure 6.2 Molecular analysis, amplification and subcloning of $\beta 5$ cDNA

(a) Restriction analysis of pcDNAneo1- $\beta 5$: (1) uncut; (2) Xba I/Xho I liberating $\beta 5$. Restriction analysis of $\beta 5$: (3) Bam HI, no cut; (4) Bst XI, cut; (5) Hind III, cut (6.2.1/2).

(b) PCR amplification of $\beta 5$ from pcDNAneo1- $\beta 5$: (1) no template; (2/4) full length $\beta 5$; (3/5) ectodomain $\beta 5$; (1-3) at 67°C; (4/5) at 68°C (6.2.3). Full length $\beta 5$ generated at 68°C termed EXCYp (4); ectodomain $\beta 5$ generated at 67°C termed EXTMp (3).

(c/d) Bam HI digest of pcDNA2-EXCYp (c) and -EXTMp (d) to liberate $\beta 5$ fragments EXCYbm (~2.4Kb) and EXTMbm (~2.15Kb; 6.2.4) for subcloning into pAcSecG2T.

(e-h) Restriction digests of pAcSecG2T-EXCYbm and -EXTMbm confirmed cloning and orientation. Expected product sizes (Kb) were 2.4, 8.6 (e; EXCY) or 2.15, 8.6 (f; EXTM) with Bam HI; 1, 2.4, 7.6 (g; EXCY) or 1, 2.1, 7.6 (h; EXTM) with Hind III.

6.2.4 Cloning of PCR products for verification of engineered Bam HI sites

The full length and ectodomain $\beta 5$ DNA fragments generated by PCR (termed EXCYp and EXTMp respectively) were ligated into the TA cloning vector pcDNA2 which was subsequently transformed into TOP10F' *E. coli*. Colonies were selected from agar plates, amplified and plasmid DNA was extracted and purified. Recombinant clones were identified by Bam HI digestion, yielding fragments of the sizes predicted for full length (~2.4Kb, termed EXCYbm) and the ectodomain (~2.15Kb, termed EXTMbm) of $\beta 5$ (Figures 6.2c/d). A large scale Bam HI digest of pcDNA2-EXCYbm and pcDNA2-EXTMbm was carried out yielding $\beta 5$ DNA fragments which were excised from a gel, and purified ready for ligation into pAcSecG2T at the Bam HI site of the MCS.

6.2.5 Ligation of $\beta 5$ DNA fragments into the baculovirus transfer vector

Purified pAcSecG2T was linearised by digestion with Bam HI, phosphatase treated to prevent self-ligation and ligated with either EXCYbm or EXTMbm. Plasmids were transformed into TOP10F' bacteria. Transformants were picked from agar plates and amplified. Purified plasmid DNA from 20 colonies in each case was digested with Bam HI to confirm ligation and Hind III to determine the insert orientation (Figures 6.2e-h). Correctly orientated inserts digested with Hind III produced DNA fragments of ~1Kb, ~2.4Kb and ~7.6Kb for pAcSecG2T-EXCYbm and ~1Kb, ~2.1Kb and ~7.6Kb for pAcSecG2T-EXTMbm. A single clone of each type was selected to be used to co-transfect SF9 insect cells (2.3.2), see Figure 6.1.

6.2.6 Partial sequencing of plasmids pcDNA2-EXCYp and pcDNA2-EXTMp

In order to confirm that following PCR the two amplified DNA fragments corresponded to the mouse $\beta 5$ integrin subunit, pcDNA2-EXCYp and pcDNA2-EXTMp were partially sequenced using the M13 forward and reverse primers. Each sequencing reaction gave between 300 and 600 bases of sequence which are shown aligned against the human $\beta 5$ integrin sequence in Figure 6.3.

Figure 6.3 Partial sequencing of $\beta 5$ -plasmids aligned against human $\beta 5$

| | |
|-------|--|
| Mouse | <u>ATGCCCGGGTGC</u> <u>CGCGACCTCTACGCCTGCTGCTCGGGCTCTGCGCGCTCGTTC</u> |
| Human | 30 <u>ATGCCCGGGGCCCGCGCGCTGTACGCCTGCCTCCTGGGGCTCTGCGCGCTCCTGC</u> 87 |
| | CGCGCTCGCAGGGCTCAACATATGCACTAGTGAAGTGCCACCTCGTGTGAAGAATGCC |
| | 88 <u>CCCGGCTCGCAGGTCTCAACATATGCACTAGTGAAGTGCCACCTCATGTGAAGAATGTC</u> 147 |
| | TGTTGATCCACCCAAAATGTGCTGGTGTCCAAAGAGTACTTTGGCAATCCACGTCCTCA |
| | 148 <u>TGCTAATCCACCCAAAATGTGCTGGTGTCCAAAGAGGACTTCGGAAGCCACGTCCTCA</u> 207 |
| | TCACCTCTCGGTGTGACCTGAAGGCCAAACCTCATCCGGAATGGCTGTGAAGGTGAGATTG |
| | 208 <u>TCACCTCTCGGTGTGATCTGAGGGCAAACCTGTCAAAAATGGCTGTGAGGTGAGATAG</u> 267 |
| | AGAGTCCAGCCAGCAGCACCACGTCCTCCGGAACCTACCTCTCAGCAGCAAGGGTTCCA |
| | 268 <u>AGAGCCAGCAGCAGCTTCCATGTCTGAGGAGCCTGCCCTCAGCAGCAAGGGTTCCG</u> 327 |
| | GTGCCACGGGCTCTGACGTCATCCAGATGACCCGCGAGAGATTGCACTGAGCTCCGGC |
| | 328 <u>GCTCTGAGGCTGGGACGTCATTGAGATGACACCAGGAGATTGCCGTGAACCTCCGGC</u> 387 |
| | CAGGCGAGCAGACTAC TCCAGCTGCAAGTGCGCCAGGTGGAAGA |
| | 388 <u>CCGGTGACAAGACCAC</u> 403 406 <u>TCCAGCTACAGGTTGCCAGGTGGAGGA</u> 433 |
| | GGGAGAACCAGGAGCGGNTACCAGAACCTTTN |
| | 1536 <u>GGGAGAACCAGGAGCGGTACACAGAACCTGTG</u> 1567 |
| | CGGGGAGGAGAGGGCAAGCCTNTGTGCAGCGGGCGTGGAGAGTGTAGCTGCAACCCAGTG |
| | 1568 <u>CCGGGAGGAGAGGGCAAGCCACTGTGCAGCGGGCGTGGGAGTGCAGCTGCAACCCAGTG</u> 1627 |
| | CTCTTGCTTCGAGAGTGAGTTCGGGAGAATCTACGGACCTTTCTGCGAGTGTGACAGCTT |
| | 1628 <u>CTCCTGCTTCGAGAGCGAGTTTGGCAAGATCTATGGGCCCTTCTGTGAGTGCACAACTT</u> 1687 |
| | TTCTGTGCCAGAAACAAGGGCGTCTATGCTCAGGCCATGGAGAGTGTCACTGTGGAGA |
| | 1688 <u>CTCCTGTGCCAGGAACAAGGGAGTCTCTGCTCAGGCCATGGCAGTGTCACTGTGGGGA</u> 1747 |
| | ATGCAAAATGCCACGAGGTTACATTGGGGACAATTGTAAGTCTCAACAGACGTACAGCAC |
| | 1748 <u>ATGCAAGTGCCATGCAGGTACATCGGGGACAACCTGTAAGTCTGACAGACATCAGCAC</u> 1807 |
| | ATGCAAGGCCAAGGATGGGAGATCTGAGTGAACGAGCCGTTGTGCTGTGGGAGTG |
| | 1808 <u>ATGCGGGGAGAGATGGCCAGATCTGACGCGAGCGTGGGCACTGTCTGTGGGAGTG</u> 1867 |
| | CCAGTGACAGAGCCTGGAGCCTTTGGGAGAGCGTGTGAGAAGTGCCCAACCTGCCCGGA |
| | 1868 <u>CCAATGCACGAGGCGGGGGCTTTGGGAGAGTGTGTGAGAAGTGCCCACTGCCCGGA</u> 1927 |
| | TGCTTGCACTCTAAGAGAGACTGTGTGAATGCTTGCTACTTCACAGGGGAACCTGA |
| | 1928 <u>TGCATGCAGCACCAGAGAGATTGCGTCGAGTGCCTGCTGCTCCACTCTGGGAACCTGA</u> 1987 |
| | CAACAGACCTGCCACACCAAGTGAAGATGAGTGTACGTTGGGTAGACACCATCGT |
| | 1988 <u>CAACAGACCTGCCACAGCCTATGCAGGGATGAGGTGATCAGTGGGTGGACACCATCGT</u> 2047 |
| | CAAAGATGACAGGAGGCTGTGCTTTGCTTCTACAAAATGCTAAGGACTGCGTTATGAT |
| | 2048 <u>GAAAGATGACAGGAGGCTGTGCTATGTTCTACAAAACCGCAAGGACTGCGTCATGAT</u> 2107 |
| | GTTCAGCTACACAGAATGCCAATGGGAGGTCCAACCTGACGGTCTCCGGGAGCCAGA |
| | 2108 <u>GTTCACCTATGTGAGGCTCCCGAGTGGGAAGTCCAACCTGACCGTCTCAGGGAGCCAGA</u> 2167 |
| | ATGTGGAAGTGCCCCAATNCNNAGACCATCCAGGTGGNNGTGGTNGACNGCATCTCNT |
| | 2168 <u>GTGTGGAACACCCCAAGCCATGACCATCTCTGGCTGTGGTGGTGTAGCATCTCTCT</u> 2227 |
| | NATAGGGATGGCACTCGTGGCCATCTGGAAGCTGATAGTNACCATCCACGACCGCCGAGA |
| | 2228 <u>TGTTGGGCTTGCACTCTGGCTATCTGGAAGCTGCTTGTACCATCCACGACCGGAGGA</u> 2287 |
| | GTTTGCCAAAGTCCCAAGTGAGCGNTCCAGGGCCGTTATGAAATGGCTCAAACCCCT |
| | 2288 <u>GTTTGCAAGTTTCAGAGCGAGCGATCCAGGGCCGCTATGAAATGGCTTCAATCCATT</u> 2347 |
| | GTACAGAAAGCCCATCTCCACACACACNGTNGATTTCGCCTTCAACAANTCAACAATGG |
| | 2348 <u>ATACAGAAAGCCTATCTCCACGCACACTGTGGACTTCACCTTCAACAATCTTACAATGG</u> 2407 |

[Primer annealing sites for amplification of sequence encoding $\beta 5$ ectodomain used for fusion protein production are underlined (forward primer 'EX' bases 7-28, reverse primer 'TM' bases 111-128). Sequence encoding transmembrane domain is denoted by a waved underline; McLean *et al.*, 1990]

Part 2 - Production, selection and amplification of recombinant baculovirus**6.2.7 Co-transfection of SF9 cells to produce recombinant baculovirus****6.2.7.1 Calcium phosphate co-precipitation-mediated co-transfection**

Introduction of DNA sequences encoding either full length or the ectodomain of mouse $\beta 5$ integrin subunit and linearised baculovirus DNA into SF9 cells was first attempted by conventional co-precipitation of the DNA using calcium phosphate. Following a standard method for this procedure as described in the manufacturer's instructions (2.3.2.1), uninfected, wild-type virus-infected and $\beta 5$ /baculovirus co-transfected cells were observed for 6 days. On the fourth day of infection negative control uninfected cells (Figure 6.4a) had continued to divide, remaining attached to the dish and on day six they appeared small, round and healthy. Positive control cells infected with a wild-type virus stock were full of polyhedrin particles by day 4 (Figure 6.4b) and by the sixth day were mostly dead having undergone lysis as expected. Immediately following co-transfection, cells exposed to the calcium phosphate precipitate appeared generally unhealthy with a granular cytoplasm. By day 4 many cells had died and the remaining cells were very swollen, but they were still attached to the dish, and showed some evidence of further cell divisions (Figure 6.4c). By day 6 of infection the cells of the sparse co-transfected dish appeared unhealthy and some cells had undergone lysis (an indication of baculovirus infection; Figure 6.4d).

6.2.7.2 Virus titre of calcium phosphate co-transfection supernatant

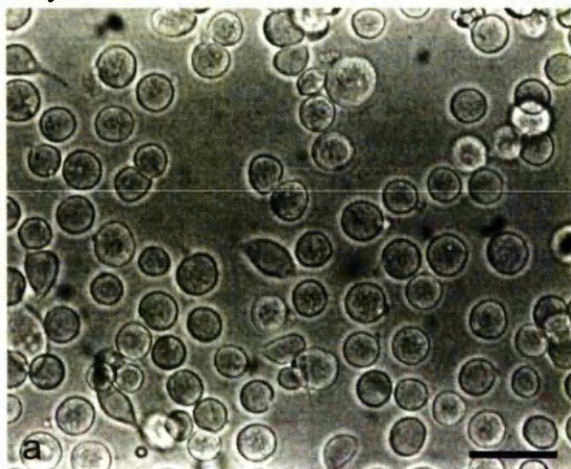
The culture supernatants resulting from calcium phosphate mediated co-transfection were collected for determination of virus titre, by plaque assay (2.3.3). At a certain dilution of culture supernatant, discrete areas of white dead cell 'plaques' should be seen by eye within the monolayer of live red cells following neutral red staining of the plaque assay plates.

Cells mock-infected with negative control supernatant were all stained red, with no white areas, demonstrating an absence of virus in the culture. Supernatant from wild type virus infected cultures served as a positive control for the plaque assay method. Here, an increasing plaque area correlated with an increasing concentration of virus inoculum, allowing calculation of virus titre. No dead cell

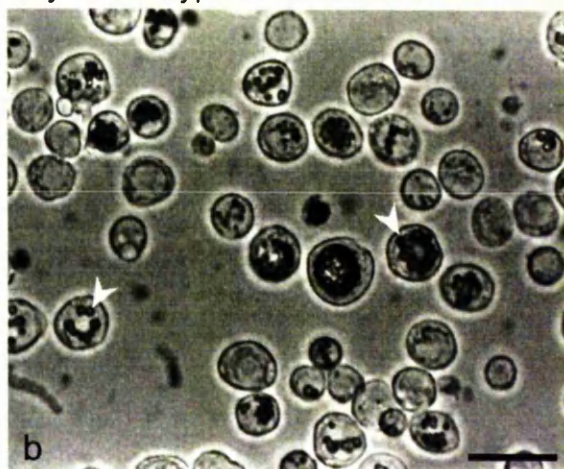
plaques were seen at any dilution of EXCY or EXTM recombinant virus supernatant by plaque assay. Also by microscopic examination most cells were stained red with only a few unstained, and therefore infected, cells scattered throughout the monolayer. This suggested that the co-transfection had not produced enough recombinant virus particles to be detected by plaque assay. Therefore the co-transfection by calcium phosphate precipitation was repeated using fresh transfer vector plasmid DNA purified from alternative clones and the resulting supernatants were again analysed by plaque assay. As before, recombinant virus co-transfection supernatants produced no plaques with only a few dead cells seen within monolayers under microscopic examination.

End point dilution assay, an alternative and less accurate method for calculation of virus titre was modified to determine simply the ability of the recombinant virus supernatants to infect SF9 cells. Multiple aliquots of SF9 cells were mixed with 10-fold dilutions of recombinant virus supernatant, negative control supernatant or wild type virus and seeded into wells of a 96-well plate as described in section 2.3.3.4. Daily examination of the cells by light microscopy allowed the course of virus infection to be monitored. By day 6 of infection only one of the ten wells, containing neat co-transfection supernatant of either EXCY or EXTM recombinant baculovirus, contained cells showing signs of infection. In these wells only 2 or 3 of the approximately 2×10^5 cells appeared infected and undergoing lysis (Figures 6.4e/f). This assay demonstrated that production of recombinant virus particles following calcium phosphate precipitation mediated co-transfection was very inefficient, resulting in a virus supernatant of little use in further infections.

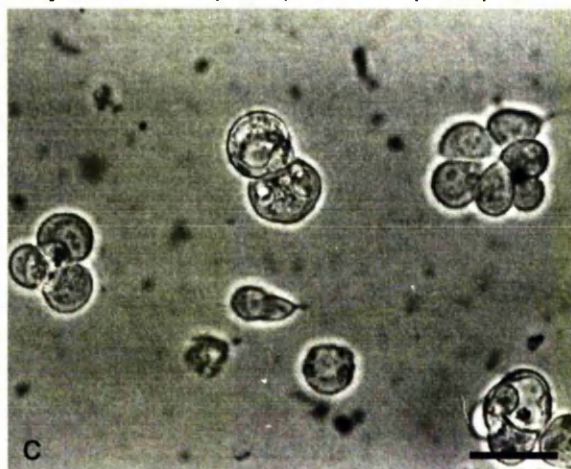
Day 4 uninfected



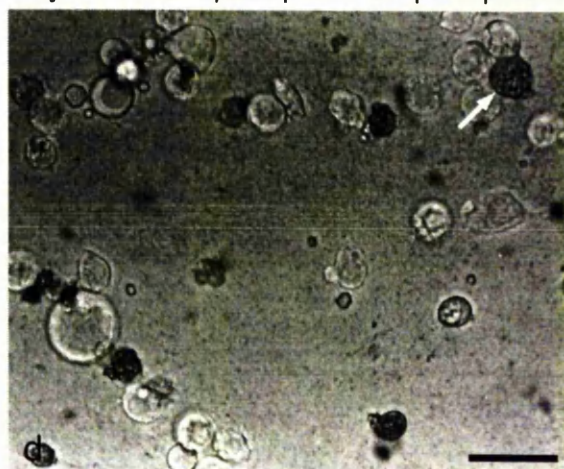
Day 4 wild-type virus infected



Day 4 calcium phosphate co-precipitation



Day 6 calcium phosphate co-precipitation



Day 6 end point dilution assay of EXCY



Day 6 endpoint dilution assay of EXTM

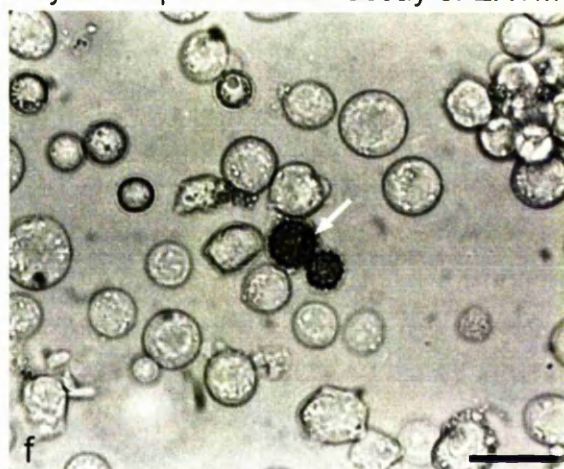


Figure 6.4 Co-transfection of SF9 cells by calcium phosphate co-precipitation

Cells exposed to calcium phosphate (c/d) appear unhealthy (see 6.2.71)

Co-transfection supernatant is of low virus titre (see 6.2.7.2), infecting few cells (e/f)

Lysed infected cells (→) and polyhedra (➤) are indicated; scale bar = 10µm

6.2.7.3 Lipofectin mediated co-transfection

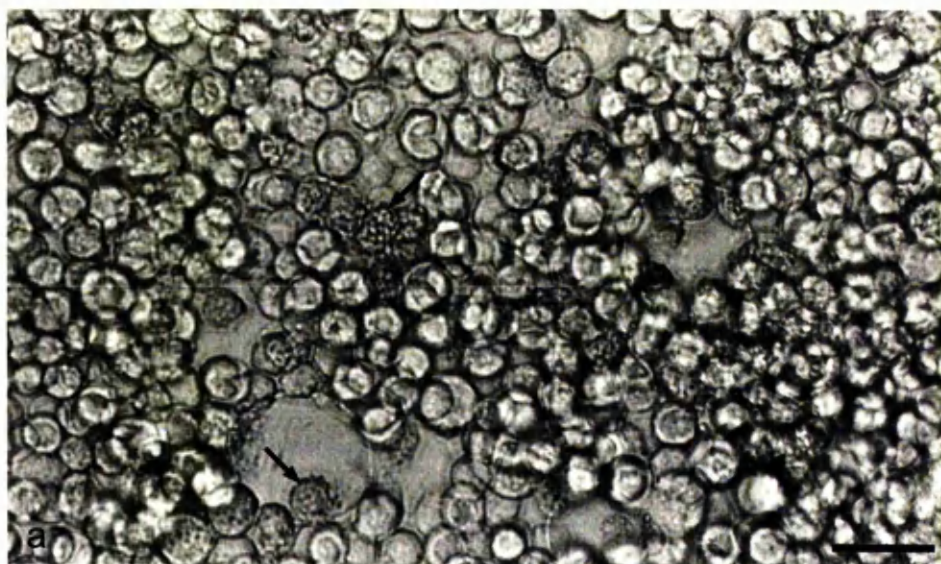
A more recent alternative method of co-transfection using a cationic lipid reagent (Lipofectin, section 2.3.2.2) for introduction of DNA into the insect cells was then employed. Immediately after co-transfection, insect cells appeared morphologically normal, without the granular cytoplasm seen following exposure to calcium phosphate as described above (6.2.7.1). On day 4 of infection at least 10% of co-transfected cells were swollen and floating, by day 6 of infection these cells had lysed and therefore showed all the predicted signs of recombinant baculovirus infection (Figure 6.5a). Negative and positive controls cells on day 6 of infection are also shown (Figures 6.5b/c).

6.2.7.4 Virus titre of Lipofectin mediated co-transfection supernatant

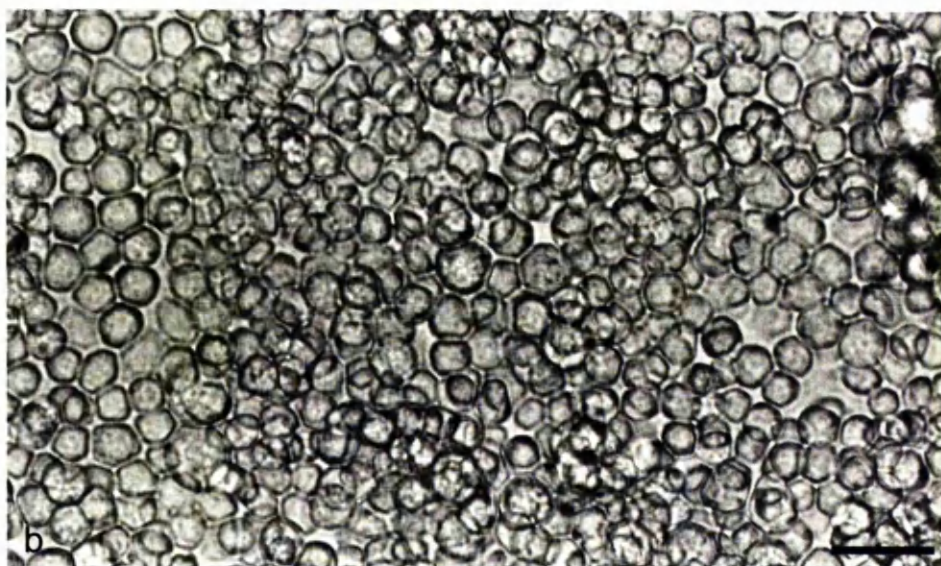
Plaque assays of culture supernatant generated by lipofectin mediated co-transfections, containing recombinant baculovirus encoding the full length and ectodomain of $\beta 5$ were carried out. Discrete plaques (10 to 40) were seen when EXCY was undiluted and EXTM was diluted 1:1000. Virus titre was then calculated (Table 6.1), in plaque forming units per ml (PFU/ml) by taking into account the number of plaques, virus dilution factor and the volume of inoculum added to the plate (standard at 0.15mls) as described in section 2.3.3.3. For example:

$$\begin{aligned}\text{EXCY} &= 30 \text{ plaques} \times 1 \text{ dilution factor} \div 0.15\text{mls standard inoculum volume} \\ &= 200 \text{ PFU/ml}\end{aligned}$$

$$\begin{aligned}\text{EXTM} &= 10 \text{ plaques} \times 1 \times 10^3 \text{ dilution factor} \div 0.15\text{mls standard inoculum volume} \\ &= 67 \times 10^3 \text{ PFU/ml}\end{aligned}$$



Day 6 recombinant baculovirus infected



Day 6 uninfected



Day 6 wild type virus infected

Figure 6.5 Co-transfection of SF9 cells using Lipofectin

Cells exposed to Lipofectin (a) that become infected undergo lysis (—→). Uninfected (b) and wild-type virus infected (c) cells are also shown (6.2.7.3). Scale bar = 10 μ m

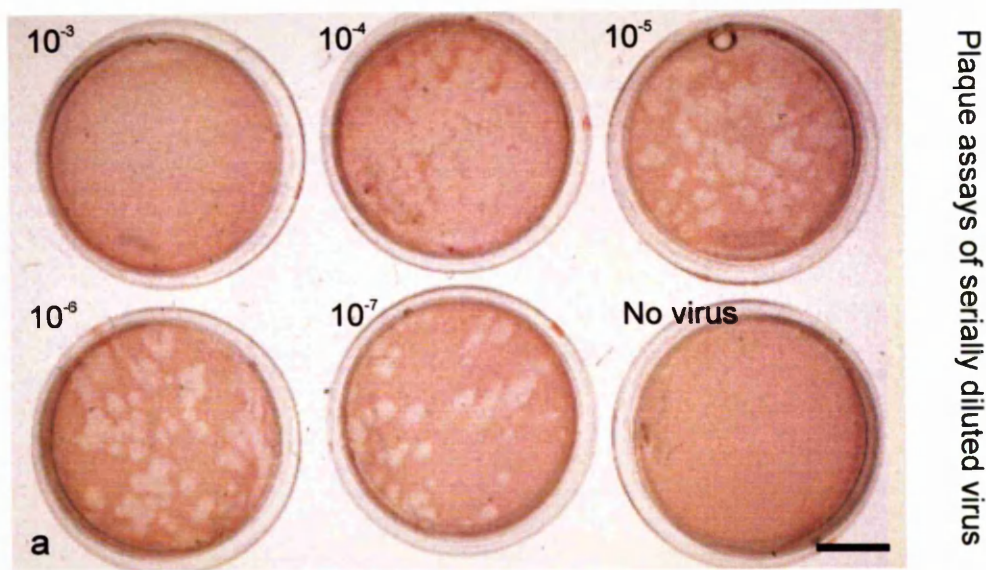
Table 6.1 Calculation of virus titre by plaque assay

| Plaque assay name | Virus supernatant assayed | Plaques counted | Virus dilution factor | Virus titre (PFU/ml) |
|-------------------|---------------------------|-----------------|-----------------------|----------------------|
| Positive | Wild type virus | 20 | 10^5 | 13×10^6 |
| EXTM 1 | Co-transfection | 10 | 10^3 | 67×10^3 |
| EXTM 2 | Seed stock | 30 | 10^6 | 20×10^6 |
| EXTM 3 | Working stock | 40 | 10^7 | 27×10^8 |
| EXCY 1 | Co-transfection | 30 | 1 (undiluted) | 2×10^2 |
| EXCY 2 | Seed stock | 40 | 10^6 | 27×10^7 |
| EXCY 3 | Working stock | 20 | 10^{10} | 13×10^{11} |

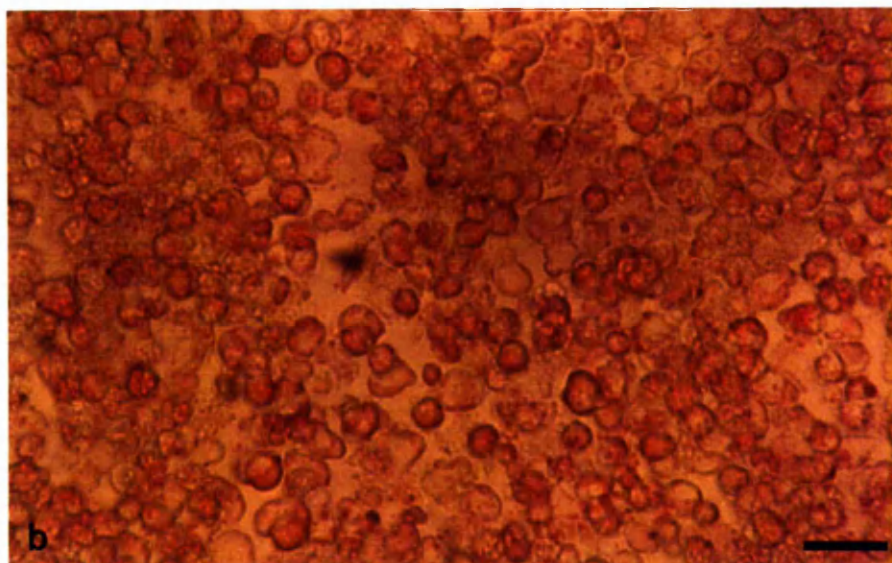
6.2.8 Selection and amplification of recombinant baculovirus clones

In order to prepare high titre (at least 1×10^9 PFU/ml) recombinant virus stocks for use in fusion protein production, amplification of co-transfection supernatant was required. For this, single virus clones were isolated by picking plaques from the first round plaque assay (EXCY1 and EXTM1) and virus particles were eluted into a small volume of medium. This first round plaque pick was subsequently used to infect cells (Table 2.4), resulting in a higher titre virus supernatant termed the 'seed' stock. The virus titre of this first amplification seed stock was determined by a second plaque assay (Table 6.1). A typical plaque assay as seen by eye (Figure 6.6a) and under light microscopy (Figures 6.6b/c) is shown, taken from the second round plaque assay of EXCY seed stock.

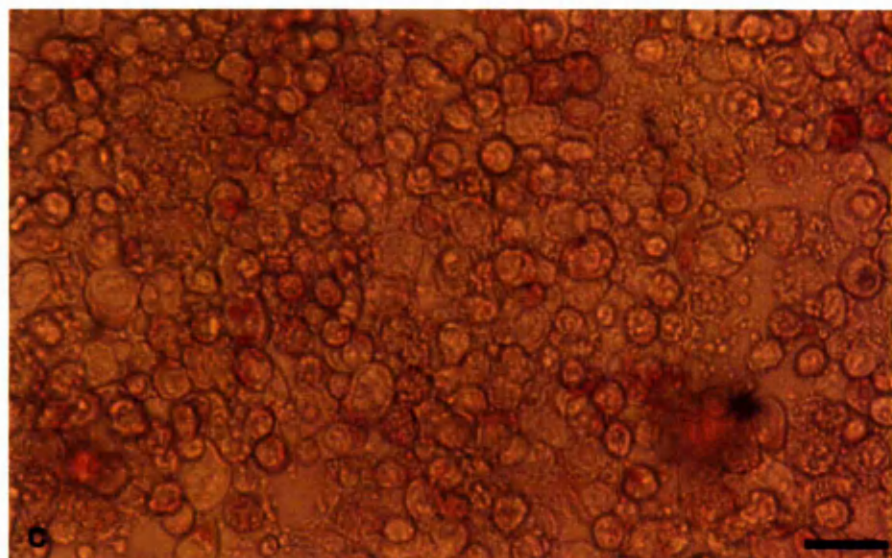
Amplification of the seed stock was then carried out as above, using a plaque picked from the second round plaque assay to infect cells (Table 2.4) resulting in an even higher titre virus supernatant termed the 'working' stock. The virus titre of this second amplification working stock was determined by a third plaque assay (Table 6.1). The working stocks of EXCY and EXTM recombinant baculovirus were at a titre appropriate for use in fusion protein production, at approximately 1×10^{12} PFU/ml and 3×10^9 PFU/ml respectively.



Plaque assays of serially diluted virus



Uninfected cells



Recombinant baculovirus infected cells

Figure 6.6 Estimation of virus titre by plaque assay

(a) Serial dilution of virus allowed visualisation of lysed cell plaques (6.2.7.4). When stained with neutral red, uninfected cells retained dye (b) while plaques of infected lysed cells remained colourless (c). Scale bar = 1cm (a); = 10 μ m (b/c).

Part 3 - Extraction of DNA and protein for analysis and purification

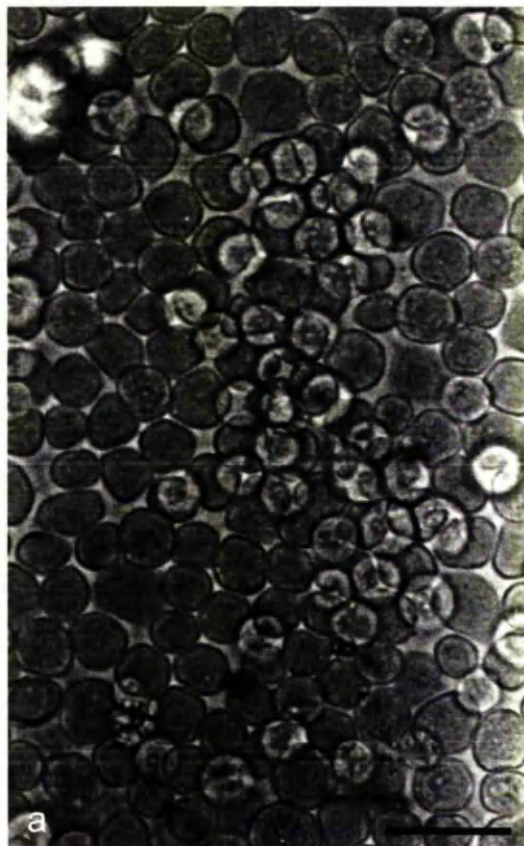
6.2.9 Morphological features of baculovirus-infected SF9 insect cells

SF9 insect cells infected with baculovirus display several features which distinguish them from uninfected cells on examination under bright field microscopy. Uninfected, recombinant baculovirus-infected and wild type baculovirus-infected cells were first observed immediately following infection (day 1) and then daily up to day 6. The features of each cell population are summarised in Table 6.2, where the stage at which a feature is first obvious is also shown. By day 4 of infection, recombinant baculovirus-infected cells can be clearly distinguished from uninfected controls (Figure 6.7). Uninfected cells continue to divide and become over-confluent (Figure 6.7a), all cells are strongly adherent to the culture dish. Any floating cells seen are due to overcrowding and when transferred to a fresh dish they will rapidly attach. The recombinant baculovirus-infected cell population is sparse, where cell numbers are similar to initial seeding density. These cells float and are approximately 50% larger than uninfected cells (Figure 6.7b). They appear transparent and nuclei are obvious, being swollen and irregular.

6.2.10 Examination of recombinant baculovirus-infected SF9 cell genomic DNA for sequence encoding mouse $\beta 5$ integrin subunit

To confirm the presence of DNA encoding either the full length or ectodomain of $\beta 5$, SF9 cell DNA was extracted (2.3.5) on day 6 of infection. PCR was carried out using the forward primer EX in combination with either reverse primer CY or TM, to amplify the full length or ectodomain of $\beta 5$ respectively. Products of the expected size for full length (~2.4Kb) and the ectodomain (~2.15Kb) of $\beta 5$ are shown in Figure 6.7c alongside corresponding products from the original $\beta 5$ plasmid DNA, pcDNAneo1- $\beta 5$.

Day 4 uninfected SF9 cells



Day 4 of recombinant virus infection

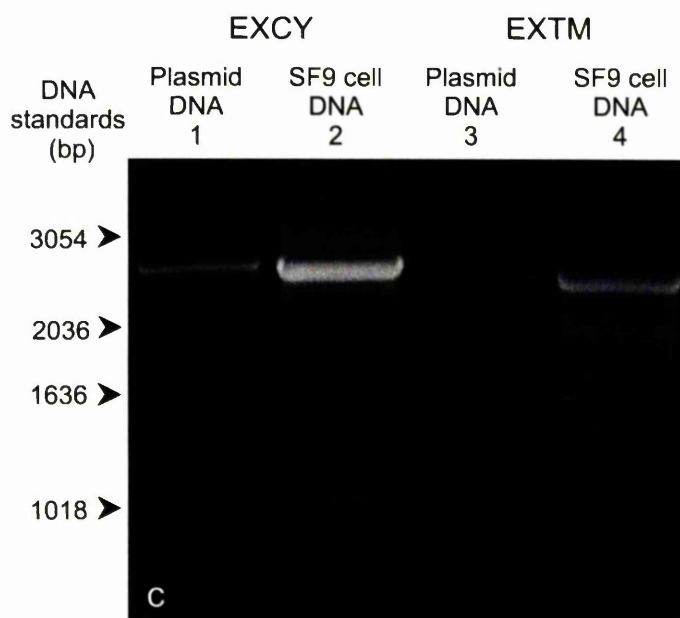


Figure 6.7 Morphological and genetic evidence of recombinant virus infection

Day 4 of infection, uninfected cells (a) continue division. Baculovirus infected cells cease growth, become swollen and float (b). See 6.2.9; scale bar = 10 μ m

(c) PCR amplification of full length $\beta 5$ (~2.4Kb; lanes 1/2) at 67°C, and ectodomain $\beta 5$ (~2.15Kb; lanes 3/4) at 68°C. Templates were pcDNAneo1- $\beta 5$ plasmid (lanes 1/3) or day 6 baculovirus-infected SF9 cell DNA extracts (lanes 2/4); see 6.2.10.

Table 6.2 Morphological signs of baculovirus infection of SF9 cells

| Feature | Uninfected | Recombinant virus | Wild type virus |
|----------------------|------------|----------------------|--------------------|
| Cell division | ✓ | x day 2 | x day 2 |
| Cell size | ↔ | ↑ day 3 | ↑ day 2 |
| Adherent | ✓ | x day 3 | x day 2 |
| Polyhedrin particles | x | x | ✓ day 3 |
| Nuclear swelling | x | ✓ day 3 | ✓ day 4 |
| Cell number | ↑ | ↓ day 6 | ↓ day 4 |
| Cell lysis | x | ✓ day 6 | ✓ day 4 |

[✓ yes; x no; ↑ increased; ↓ decreased; ↔ unchanged]

Cells were scored daily, and for infected cells the day a morphological change was first observed was recorded.

6.2.11 Analysis of proteins produced by SF9 cell cultures

6.2.11.1 Following the time course of fusion protein production

Small scale cultures of EXCY and EXTM recombinant baculovirus-infected SF9 cells were maintained in complete growth medium. Cellular protein was extracted in the presence of SDS, 24 to 96 hours post-infection on days 2, 3, 4 and 5 of infection (section 2.3.6). In addition, protein was extracted from mock-infected negative control cells at equivalent time points. Protein concentrations of samples were assayed and normalised prior to separation of 25 μ g samples on polyacrylamide gels.

Protein extracted on days 2, 3 and 4 from cells infected with EXCY or EXTM and uninfected cells was separated by SDS PAGE. Gels were stained or blotted onto nitrocellulose for chemiluminescent detection. The Coomassie-stained gel demonstrates equal loading of protein samples (Figure 6.8a). Western blotting using an anti-GST antibody detected GST-tagged fusion proteins only, that is, proteins coded for by DNA originating from the pAcSecG2T transfer vector. It can be seen that only 24 hours post-infection, fusion protein is detectable (Figure 6.8b). Levels of the 'complete' fusion protein and what seem to be various fragments tagged to GST increase on days 3 and 4 of infection (Figure 6.8c). This suggests that most $\beta 5$ fusion protein would be extracted from cells 72 hours post-infection (see Figure 6.7b). The 'complete' GST-tagged fusion protein of full length mouse $\beta 5$ is ~140kDa, the ectodomain only protein is ~129kDa and GST alone appears to be ~32kDa.

6.2.11.2 Confirmation that extracted fusion protein is $\beta 5$ integrin

An antibody directed to a 30AA sequence within the cytoplasmic domain of the human $\beta 5$ integrin subunit was used to detect full length fusion protein 24 to 96 hours post-infection by Western blotting (Figure 6.9b). Fusion protein of ~129kDa was undetectable on day 2 but was clearly seen on days 3, 4 and 5. It again appeared to be at its highest level in cell lysates 72 hours post-infection. Several bands were seen as described above using the antibody to GST.

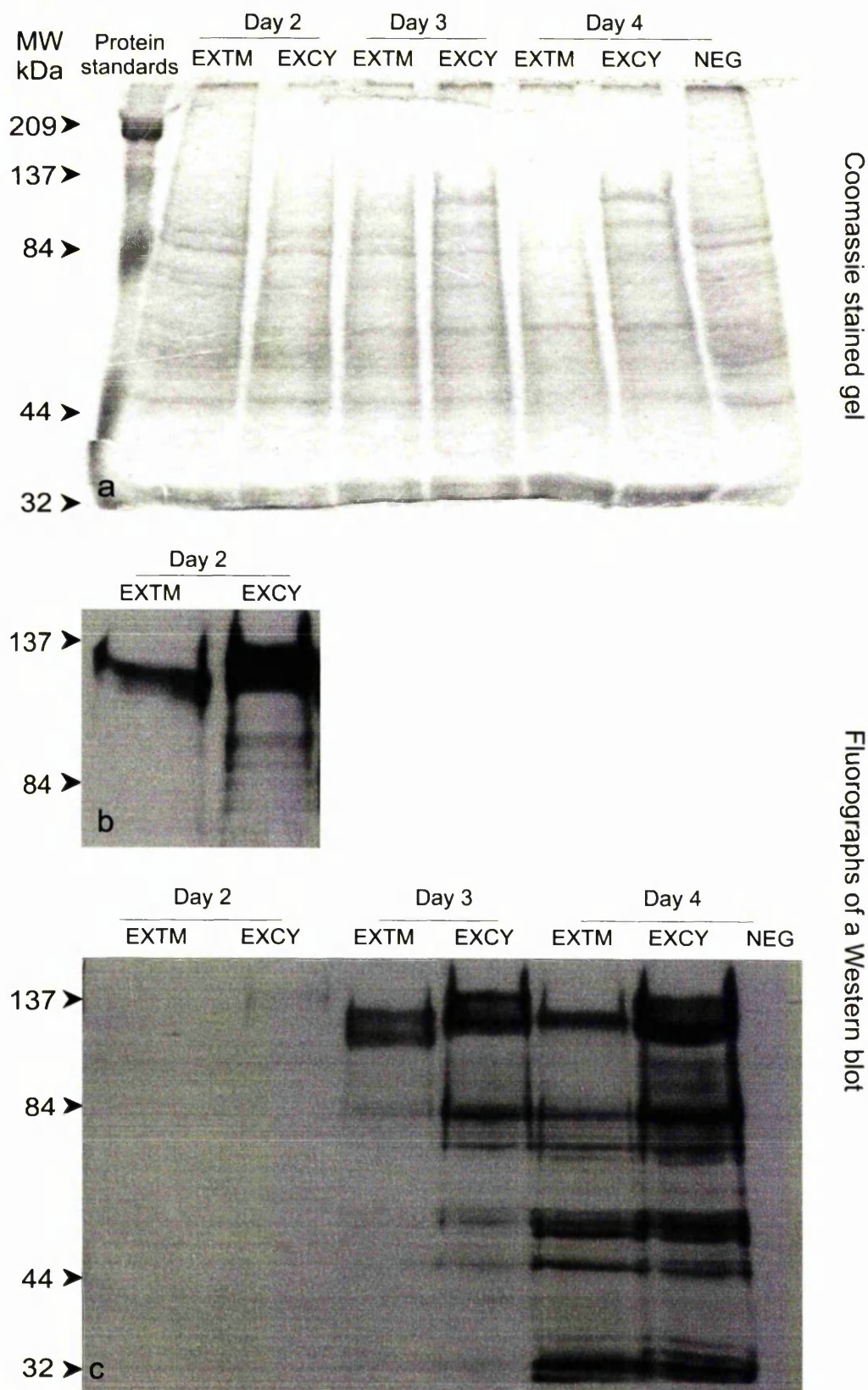


Figure 6.8 A study of the time course of fusion protein production

(a) Coomassie stained gel showing equal loading of protein extracts from cells infected with baculovirus encoding full length (EXCY) and ectodomain (EXTM) $\beta 5$, on days 2, 3, 4 of infection. Extract of uninfected cells (NEG) also shown (6.2.11.1).

(b/c) Fluorographs of a Western blot, using anti-GST to detect fusion proteins. Extended exposure permitted detection of EXCY and EXTM fusion protein on day 2 (b). Increasing levels of fusion protein were detected from day 2 to 4 (c).

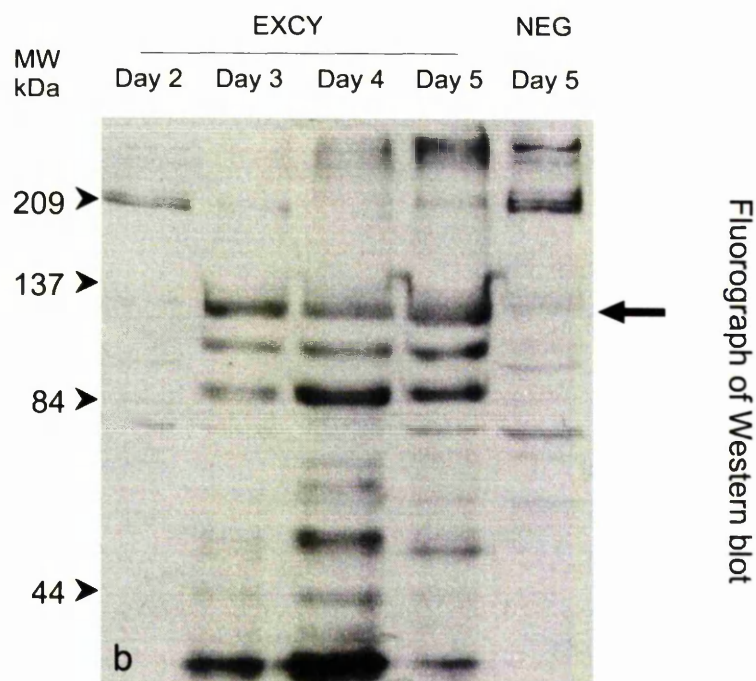
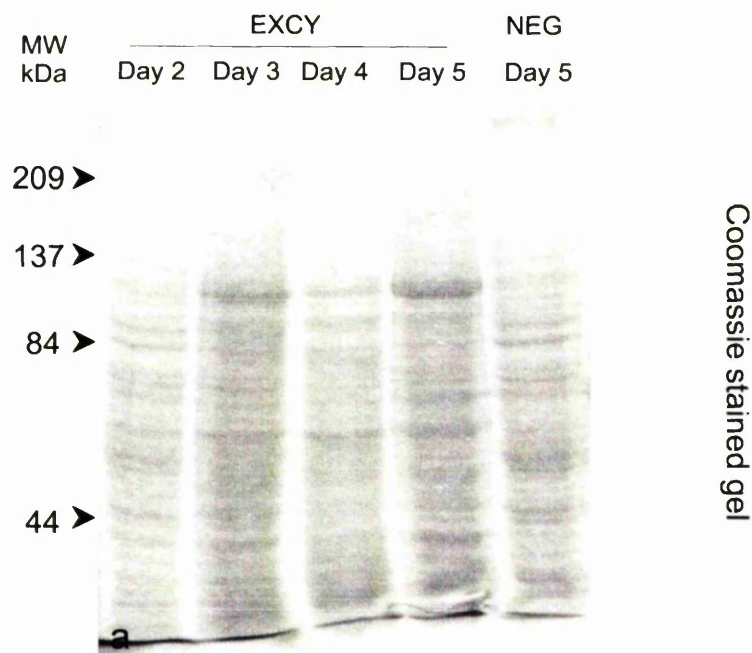


Figure 6.9 Identification of fusion protein as mouse $\beta 5$ integrin

(a) Coomassie stained gel showing equal loading of protein extracts from cells infected with baculovirus encoding full length $\beta 5$ (EXCY) on days 2, 3, 4, 5 of infection. Extract of uninfected cells (NEG) also shown (6.2.11.2).

(b) Fluorograph of a Western blot, using an antibody to the cytoplasmic tail of $\beta 5$. $\beta 5$ fusion protein was detected at increasing levels from day 3 to 5. Protein of the expected size (~ 129 kD) is indicated (→), several smaller proteins were also seen.

6.2.11.3 Secretion of fusion proteins into culture medium by SF9 cells

The baculovirus transfer vector pAcSecG2T was chosen for two important features, the GST-tag which would be attached to fusion proteins allowing their detection, and for the gp67 signal sequence which should lead to secretion of fusion protein into the culture medium thus aiding purification.

Cell conditioned complete growth medium was collected prior to cell lysis and analysed for the presence of secreted fusion protein, in order to determine at which stage the most fusion protein was being secreted into the culture medium. Twenty-five microgram samples of EXCY- and EXTM-infected cell conditioned medium on days 2, 3 and 4 were analysed by Western blotting using antibodies to GST and $\beta 5$ cytoplasmic domain (where appropriate). No fusion protein could be detected in any sample, indicating that fusion proteins were not secreted. Figures 6.10a/b show EXCY infected complete growth medium on a Coomassie gel and Western blot using the anti- $\beta 5$ antibody.

Cells were then propagated in serum-free growth medium and infected to determine whether fusion protein could be detected in cell conditioned medium in the absence of serum proteins, on days 2 to 5 of infection. Initially cells were infected with EXTM recombinant baculovirus only to produce the $\beta 5$ ectodomain fusion protein. Potentially these proteins which lack a transmembrane domain, are less likely to remain within the cells. Following collection, medium samples were concentrated twice (12- to 100-fold) to allow loading of $\geq 50\mu\text{g}$ protein onto gels. Western blotting using an antibody to the GST tag (Figure 6.10c) demonstrated that the only evidence of secreted protein ($\sim 120\text{kDa}$) was on day 3 of infection in a 14 times concentrated sample of conditioned medium, indicating that cell lysates would be a better source of fusion protein.

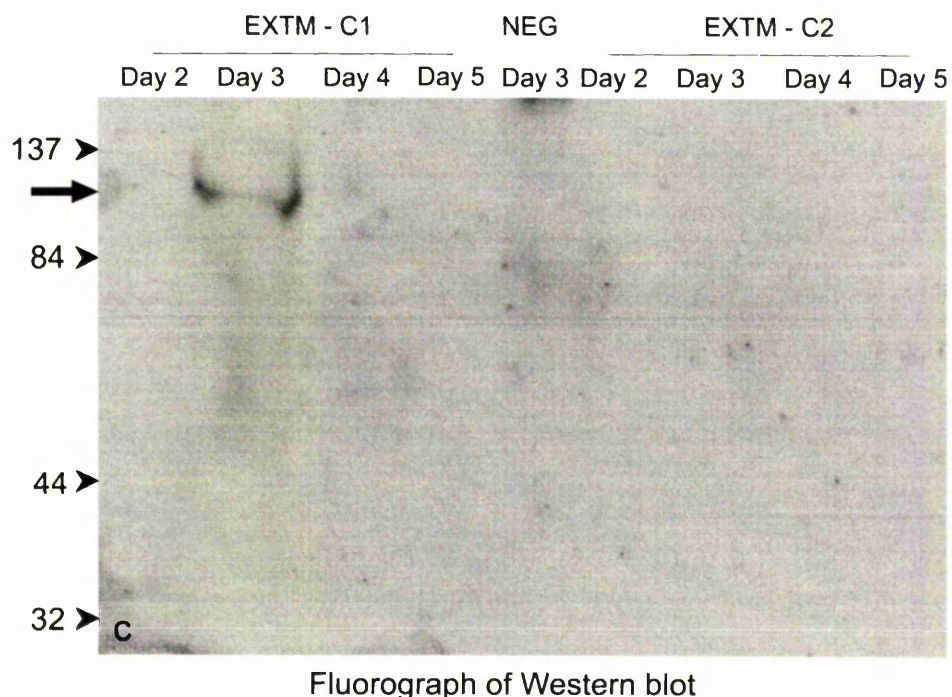
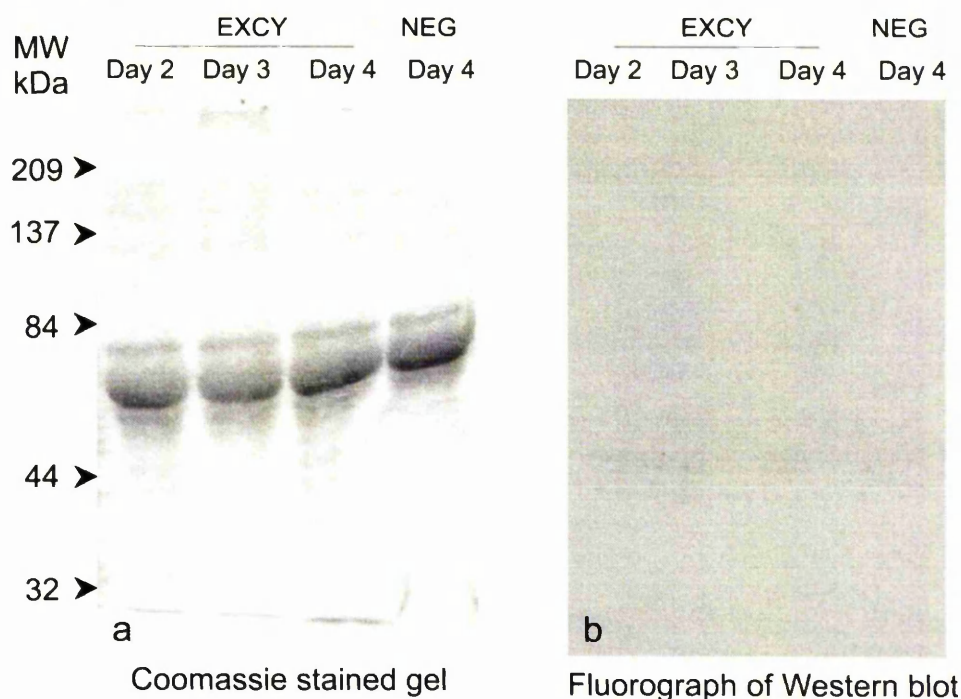


Figure 6.10 Detection of fusion protein in SF9 cell-conditioned medium

(a) Coomassie stained gel showing equal loading of cell-conditioned complete growth medium from cells infected with baculovirus encoding full length $\beta 5$ (EXCY) on days 2 to 4 of infection. Medium from uninfected cells is also shown (NEG). (b) Fluorograph of a Western blot of samples of cell-conditioned complete growth medium (as described in Figure 6.10a), using an antibody to $\beta 5$. $\beta 5$ fusion protein was not detected in any sample. (c) Fluorograph of a Western blot using anti-GST, of cell-conditioned serum-free growth medium; from cells infected with baculovirus encoding ectodomain $\beta 5$ (EXTM) on days 2 to 5 of infection. Medium from uninfected cells is also shown (NEG). Following concentration of the medium (C1, 12-35 fold) fusion protein of the expected size (~120kD) was detected on day 3 only (→). No fusion protein was detected following additional concentration (C2, 50-100 fold); see 6.2.11.3.

6.2.11.4 Extraction of protein from SF9 cells without denaturation

For purification of GST-tagged fusion proteins from cell lysates using glutathione-Sepharose beads, the enzyme activity of GST must be retained. Thus cell lysis must not cause denaturation of extracted protein and therefore cannot use SDS based lysis buffers. The efficiency of several cell lysis methods comparing non-ionic detergent with those involving SDS, by Coomassie staining of protein samples and Western blotting, is shown in Figure 6.11. Two alternative detergent-based lysis buffers were used. 'Triton X-100 lysis buffer A' (TX-A) liberated less cellular protein than 'Triton X-100 lysis buffer B' (TX-B). TX-A and TX-B contained the same amount of detergent (1%), but TX-B contained more NaCl (150mM versus 100mM), was used at a greater volume (3ml versus 2ml), and the lysate was not sonicated unlike with TX-A (2.3.7.1). Addition of 1% SDS to TX-B (TX-B+SDS; 2.3.6) extracted additional protein from detergent-insoluble stores. Fusion protein detected in cellular protein extracted using detergent alone is of a similar size to that extracted under SDS on day 2 of infection. A single protein of ~117kDa, about the size expected for the ectodomain of $\beta 5$, was extracted with Triton X-100. The remaining unlysed cell debris when extracted further in the presence of SDS, released additional 'complete' fusion protein of the expected size as well as several smaller GST-tagged fusion proteins and GST alone at ~32kDa (on day 3 of infection).

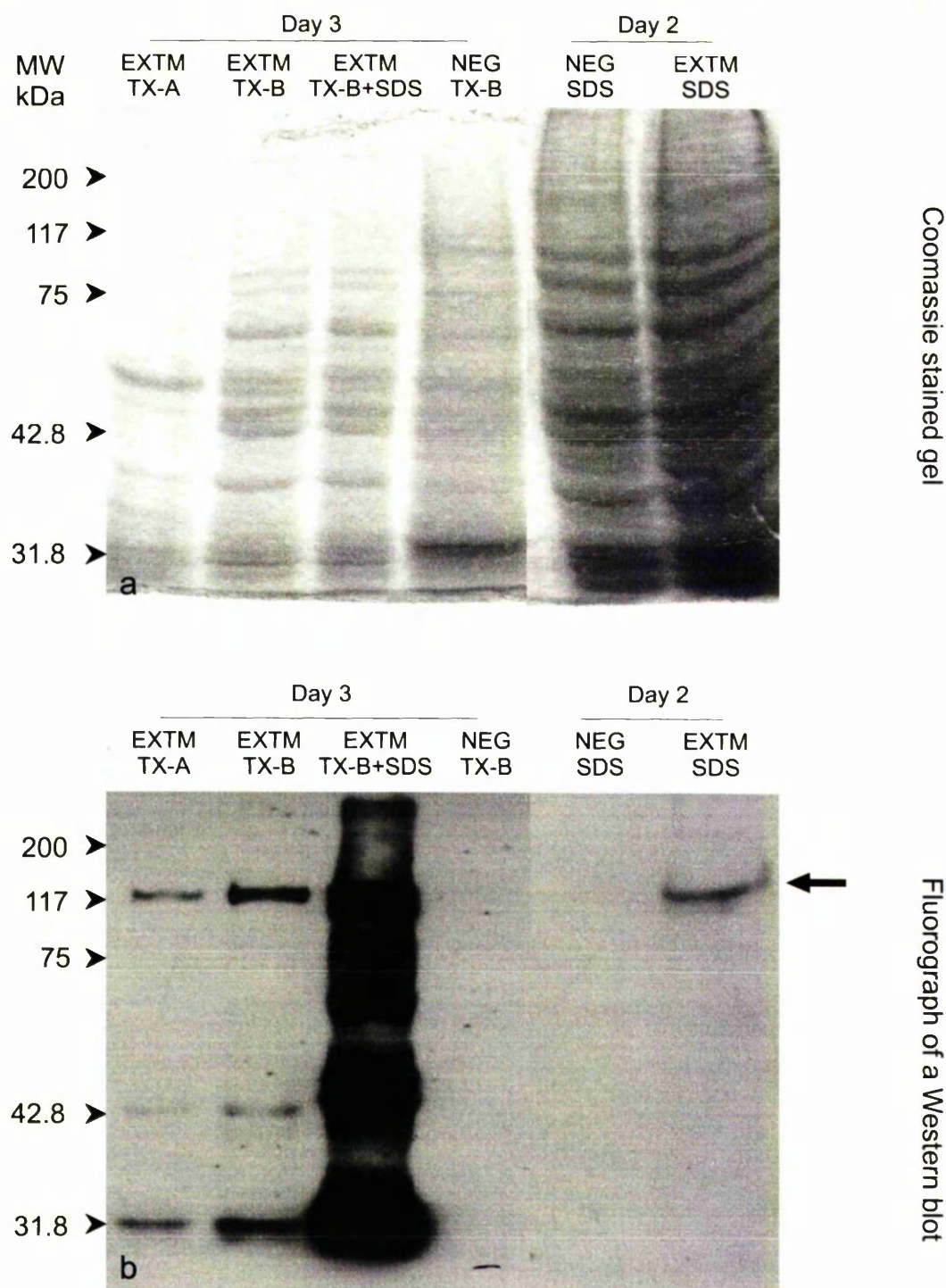


Figure 6.11 Protein extraction from SF9 cells using different lysis buffers

(a) Coomassie stained gel showing loading of protein extracted using alternative lysis buffers; from cells infected with baculovirus encoding ectodomain $\beta 5$ (EXTM). Uninfected cells are also shown (NEG). SDS-based lysis buffers (TX-B+SDS, SDS) and those using Triton-X100 detergent (TX-A, TX-B) were compared, see sections 2.3.6/7 and 6.2.11.4 for buffer-specific protocols.

(b) Fluorograph of a Western blot using anti-GST. More fusion protein was detected on day 3 than day 2 of infection. On day 3, fusion protein of a single size (~ 117 kD) was extracted with detergent (TX-A or TX-B) as indicated (\rightarrow), addition of SDS to the lysis buffer (TX-B+SDS) liberated several additional smaller fusion proteins; these smaller fusion proteins were not detected in SDS extracts on day 2.

6.2.11.5 Large scale protein production and purification

Having determined which detergent protocol to use, lysates of cells maintained in serum-free growth medium at days 2, 3, 4 and 5 of infection were prepared and analysed by Western blotting using the anti-GST antibody (Figure 6.12a). This demonstrated at which stage the best yield of ~117kDa fusion protein could be obtained, and that the time course of fusion protein production is unaffected by cell growth in the absence of serum.

To scale up fusion protein production, SF9 cells were grown at a higher density as 100ml suspension cultures. The protocol for detergent lysis was optimised to determine the required ratio of cells to buffer and the time scale (2.3.7.2). A total of 1200ml of cell suspension maintained in serum-free growth medium was infected with EXTM recombinant baculovirus. At 72 hours post-infection the cells were pelleted and lysed using TX-B, producing a total lysate volume of 380ml. glutathione-Sepharose beads were added to the lysate and incubated overnight to allow binding of the GST-tagged fusion proteins to the beads. The following day, fusion protein was eluted from the beads. Equal volumes of the three resulting eluates were loaded onto gels for silver staining and Western blotting (Figures 6.12b/c). A strong band of ~117kDa, the size expected for GST-tagged ectodomain of $\beta 5$ can be seen in the first eluate and to a lesser extent in the second. In addition smaller species likely to be ectodomain protein (~85kDa) and GST (~32kDa) alone are seen by silver staining (Figure 6.12b). The presence of GST-tagged $\beta 5$ and free GST was confirmed by Western blotting (Figure 6.12c).

A spectrophotometric (2.3.8.4) assay for quantification of the relative activity of GST between samples confirmed that 10 μ l of eluate 1 contained significantly more GST than 10 μ l of eluates 2 or 3 (Table 6.3). The protein content of eluates 1, 2 and 3 was determined using a Bradford protein assay (2.3.9.2). This indicated that eluate 1 contained ~300 μ g total protein, eluate 2 contained ~170 μ g total protein whereas the protein content of eluate 3 was negligible. The combined amount of fusion protein in eluates 1 and 2 indicated that fusion protein was present in the cellular fraction at ~400 μ g per litre of cell suspension.

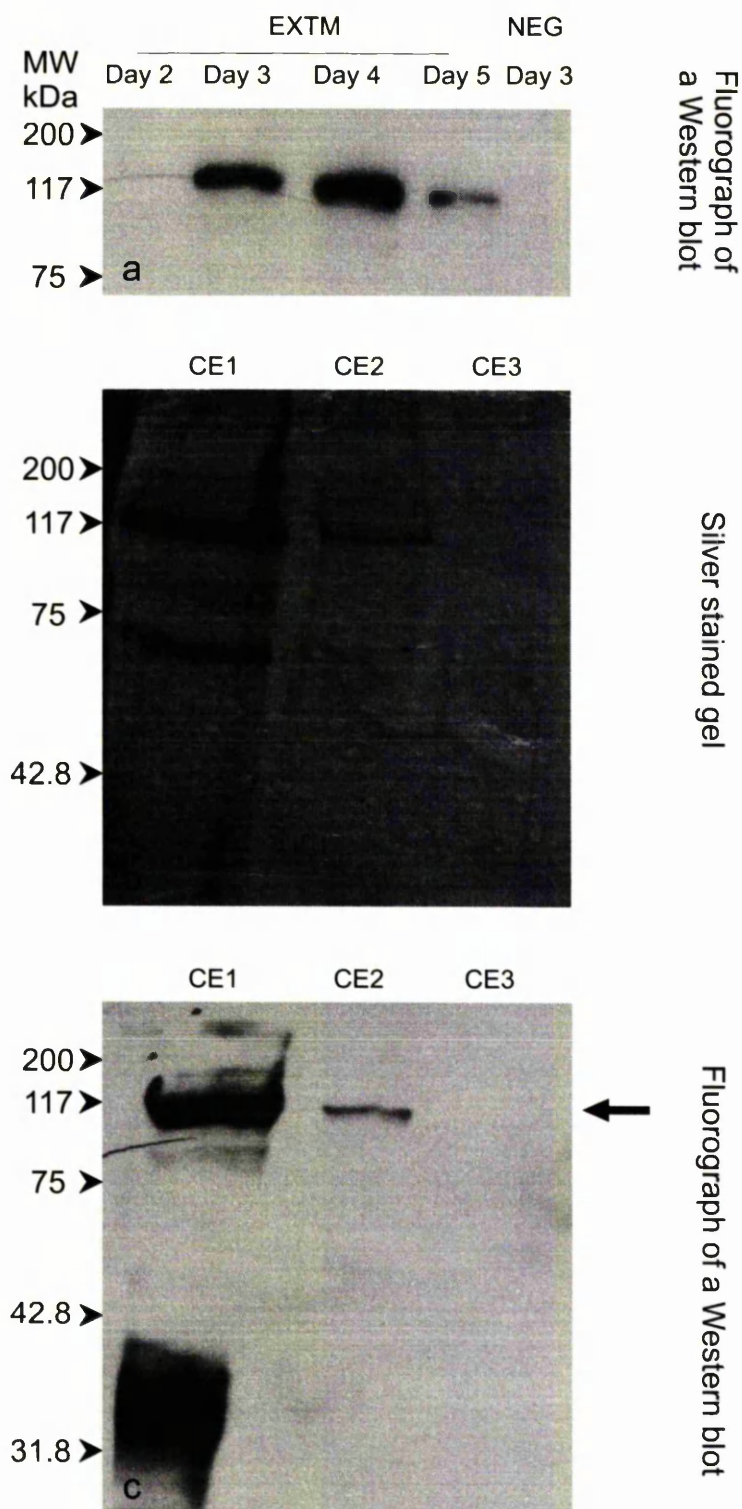


Figure 6.12 Large scale production and purification of fusion protein

(a) Fluorograph of an anti-GST Western blot, of detergent extracted (TX-B) cells infected with baculovirus encoding ectodomain $\beta 5$ (EXTM), on days 2 to 5 of infection. An extract of uninfected cells is also shown (NEG). Most fusion protein of the expected size (~117kD) was detected on day 4.

(b) A silver stained gel of fusion protein purified on a glutathione column from detergent extracts of cells infected with baculovirus encoding ectodomain $\beta 5$, at day 4 of infection. Most protein was detected in the first elution (CE1).

(c) Fluorograph of a Western blot of glutathione column eluates using anti-GST. Fusion protein of the expected size for ectodomain $\beta 5$ (~117kD) is indicated (→). Free GST (~32kD) was also detected; see 6.2.11.5.

Table 6.3 Spectrophotometric assay of relative GST activity of eluates

| Time (minutes) | Absorbance at 340nm | | |
|---------------------|---------------------|----------|----------|
| | Eluate1 | Eluate 2 | Eluate 3 |
| 1 | 0.215 | 0.012 | 0.002 |
| 2 | 0.289 | 0.018 | 0.006 |
| 3 | 0.404 | 0.022 | 0.009 |
| 4 | 0.501 | 0.033 | 0.014 |
| 5 | 0.635 | 0.043 | 0.019 |
| Δ Abs/min/ml | 10.5 | 0.8 | 0.4 |

[Δ Abs/min/ml = change in absorbance per minute per ml of sample]

6.2.11.6 Immunisation of $\beta 5$ fusion protein

Prior to immunisation, pre-immune serum was collected from 2 rabbits. Due to the limited amount of fusion protein available for immunisation, purified protein was not thrombin-cleaved, and therefore retained its GST tag. For production of polyclonal antisera ~100 μ g of purified $\beta 5$ was injected sub-dermally (1:1 in Freund's complete adjuvant). Animals were boosted with 70 μ g purified $\beta 5$ (1:1 in Freund's incomplete adjuvant) 3 months later. After a further 3 months test-bleeds were taken; these are currently being assessed for specificity and antibody titre.

6.3 DISCUSSION

To further dissect the roles of specific integrins in implantation, function-blocking antibodies specific to individual mouse β subunits are required. These are of very limited availability. Therefore the ultimate aim of the work described in this chapter was to produce an antibody directed to the mouse $\beta 5$ integrin subunit with function blocking activity. For this, fusion proteins of the mouse $\beta 5$ integrin subunit were produced in insect cells, these would then be used for immunisation. In addition, the $\beta 5$ fusion protein itself would also be available for use in integrin functional studies.

cDNA encoding mouse $\beta 5$ integrin subunit was amplified by PCR to produce full length and a truncated form (lacking transmembrane and cytoplasmic domains) for subcloning into a baculovirus transfer vector (pAcSecG2T). Partial sequencing of these fragments showed that they share ~93% identity with the corresponding regions of human $\beta 5$ cDNA (McLean *et al.*, 1990). This sequencing reaction provided an additional ~500bp of mouse $\beta 5$ cDNA sequence, encoding the membrane-proximal region of the extracellular domain, corresponding to bases 1536-2052 of human $\beta 5$ (McLean *et al.*, 1990).

Production of fusion proteins using the baculovirus/insect cell expression system has several advantages over prokaryotic, yeast or mammalian systems (King & Posse, 1992). These include: (a) dispensable virus gene products which can be substituted with those of foreign genes; (b) strong gene promoters which allow synthesis of large quantities of fusion protein; (c) promoters with activity late in the virus cycle, allowing virus replication in the presence of potentially cytotoxic fusion protein. Each of these features is common to the polyhedrin gene, which codes for a protein that is required for packaging of virus particles within 'polyhedra'. These protective coats are not necessary for virus replication under culture conditions, allowing substitution of the polyhedrin coding region with a gene of interest, whilst making use of its strong promoter. However, fusion protein expression in insect cells is discontinuous, as baculovirus infection results in cell death (King & Posse, 1992).

To maximise the chance of producing a function-blocking antibody to mouse $\beta 5$ integrin, the ligand binding regions of the fusion protein would need to reflect those of native $\beta 5$. Correct protein folding was clearly important, although the significance of integrin glycosylation in ligand binding is not fully understood. An advantage of using the baculovirus insect cell expression system was that it provides eukaryotic glycosylation, unlike a bacterial expression system. Although protein glycosylation by insect cells is different to that of vertebrates, it appeared that this system would be suitable for production of integrin fusion proteins.

In platelets, both subunits of integrin $\alpha IIb\beta 3$ contain approximately 15% carbohydrate (McEver *et al.*, 1982). A study of the processing and assembly of $\alpha IIb\beta 3$ showed that single chain precursors of both subunits have high mannose N-linked oligosaccharides added in endoplasmic reticulum. Following dimer assembly, the pre-proteins are transported to the Golgi where the high mannose chains of αIIb are modified to produce complex oligosaccharides. Therefore mature αIIb is produced with a higher molecular weight than that of its pre-protein. $\beta 3$ glycans underwent little further processing in the Golgi, remaining predominantly high mannose type chains (Rosa & McEver, 1989; Wippler *et al.*, 1994). Compared to that in other eukaryotic cells, insect cell glycosylation is relatively simple and produces oligosaccharides of high mannose type (King & Posse, 1992). Therefore the insect cell baculovirus system should be effective in the glycosylation of integrin β subunit fusion proteins. For glycoproteins that have a more complex pattern of glycosylation, insect cell fusion proteins may have greater mobility on polyacrylamide gels (lower molecular weight) than native protein (Wippler *et al.*, 1994).

When insect cells were first described as a source of fusion protein they were co-transfected with a transfer vector containing the gene of interest, and circular baculovirus (AcMNPV) DNA. One problem with this early method was distinguishing between cells that were infected with intact wild-type virus and those infected with recombinant virus. The use of linearised baculoviral DNA (Kitts *et al.*, 1990), an engineered derivative of AcMNPV baculovirus possessing a unique restriction site was a great advance in baculovirus insect cell

technology. Although linear baculoviral DNA does not co-precipitate into insect cells as efficiently as circular viral DNA, it shows a 10-fold greater chance of recombination with transfer vector DNA. Further advances came when linear baculovirus DNA was modified to incorporate regions which disabled the infectivity of the baculovirus, which could only be rescued upon recombination with the complete sequence of foreign DNA from an appropriate transfer vector. So today, of the recombinants baculoviruses achieved, approaching 100% contain the complete cDNA of interest. These advances essentially eliminated the need for further rounds of baculovirus purification. Nevertheless as an additional control, virus supernatants are routinely generated from a single virus clone derived from a plaque assay of baculovirus-infected cell-conditioned medium.

In the present study, co-transfection of insect cells was initially attempted using calcium phosphate co-precipitation of the cells. This technique was shown to be very detrimental to the cells, producing abnormal morphology and retarded growth immediately following co-transfection. An alternative approach was then taken, using a lipid reagent (Lipofectin) to stimulate DNA uptake by insect cells. This method was much better, producing no visible signs of cells damage except those expected to occur upon baculovirus infection. Due to the constraints of time, work focused on the expression of $\beta 5$ ectodomain fusion protein, which was expected to provide the best source of readily purified fusion protein. Also this fragment would contain the regions of most use for immunisation, in the production of a function-blocking antibody to mouse $\beta 5$.

Fusion proteins of integrin heterodimers have been expressed previously in insect cells, notably $\alpha v \beta 3$ (Mehta *et al.*, 1998) and $\alpha I I b \beta 3$ (Bennett *et al.*, 1993), providing valuable information regarding the synthesis and processing of integrins. Here, insect cells were co-transfected with linear baculovirus DNA and two transfer vectors each containing the sequence of a single integrin subunit. A characteristic of insect cells that are infected with recombinant baculovirus is that they become swollen and detach from the culture dish (King & Posse, 1992). However when infected with a baculovirus encoding full length $\beta 3$ integrin subunit

together with one encoding full length or truncated αv (ectodomain-only), insect cells remain attached to the culture dish and become spread (Mehta *et al.*, 1998). In these cells, fusion protein is anchored within the insect cell plasma membrane and is therefore able to mediate cell attachment to serum proteins. In the absence of the transmembrane and cytoplasmic domains of $\beta 3$ the integrin heterodimers are secreted. Therefore it is one or both of these regions which prevent secretion of the $\alpha v\beta 3$ integrin fusion protein (Mehta *et al.*, 1998).

Production of $\alpha IIb\beta 3$ fusion protein by insect cells has demonstrated that integrin dimer formation takes place in the endoplasmic reticulum (Bennett *et al.*, 1993). An excess of αIIb was expressed over that of $\beta 3$, suggesting that β subunit production was the limiting factor in integrin formation. Undimerised integrin subunits remained in the endoplasmic reticulum and were rapidly degraded (Bennett *et al.*, 1993). As seen for $\alpha v\beta 3$ (Mehta *et al.*, 1998), $\alpha IIb\beta 3$ cDNA truncated to exclude the transmembrane and cytoplasmic domains of both subunits encode fusion protein that is readily secreted (Bennett *et al.*, 1993). When insect cells are transfected with truncated cDNA encoding for a single subunit, the resulting fusion protein (ectodomain-only) of $\beta 3$ is secreted. This was due to exclusion of sequence specifically within the transmembrane domain of $\beta 3$ (Bennett *et al.*, 1993). Truncated αIIb (ectodomain-only) is retained by the cells, bound to a 115kDa protein. $\beta 3$ fusion protein can also bind this 115kDa protein, but this does not prevent secretion of this subunit. Therefore truncated αIIb appears to contain sequences within the extracellular domain that causes retention of this protein by cells, and the activity of this region is prevented by heterodimer formation. Integrin dimer formation may either displace a factor preventing αIIb secretion, or alter the configuration of αIIb to a form which can then be secreted (Bennett *et al.*, 1993).

Collectively this data regarding the synthesis of integrin fusion proteins implies that display of native integrins at the plasma membrane *in vivo* may be achieved by unique features of both the α and β subunits of integrin heterodimers. It appears that the α subunit is made to excess, and that undimerised α subunits are sequestered within cells. Dimerisation of the α subunit with an appropriate β

subunit permits release of the integrin, which is then anchored within the cell membrane by activity of the β subunit transmembrane region.

In the current study it was hoped that fusion protein could be purified from cell-conditioned culture medium, as has been reported in similar studies (Wippler *et al.*, 1994; Clark & Kulathila, 1997; Mehta *et al.*, 1998). However it was necessary to extract it from the insect cells by treatment with detergent or SDS, as fusion proteins of the mouse $\beta 5$ integrin subunit were not secreted by insect cells even when the ectodomain alone was expressed. This was unexpected as the transfer vector used for co-transfection of the insect cells contained a signal sequence (gp67) that should direct the fusion protein for secretion (King & Posse, 1992). Secretion of fusion protein has been demonstrated for other molecules, mediated by the gp67 signal sequence (Dr Nick Smithers, Glaxo-Wellcome, personal communication). So although an ectodomain-only fragment of $\beta 3$ is readily secreted (Bennett *et al.*, 1993), an equivalent construct of $\beta 5$ is not. The truncated $\beta 5$ fusion protein could not have become membrane anchored as it was not in possession of a transmembrane domain. As dimerisation is thought to be required for integrins to access the cell membrane (Bennett *et al.*, 1993), the same may be important for $\beta 5$, perhaps explaining why $\beta 5$ was retained within cells. It is possible that $\beta 5$ possesses sequences within the ectodomain (not present or functional in $\beta 3$) which prevent $\beta 5$ secretion. However human $\beta 3$ and $\beta 5$ protein sequences show 56% identity, with 70% identity in the putative RGD-binding region of the ectodomain, making them the most closely related of all integrin β subunits (McLean *et al.*, 1990). The lack of secretion of $\beta 5$ fusion protein seen in the present study could also be due to species-specific differences between the synthesis of integrins in mouse and human. Future co-expression studies using $\beta 5$ and αv constructs are necessary, to determine the fate of $\beta 5$ integrin fusion proteins.

Fusion protein was retrieved by lysis of insect cells. When cells were lysed using detergent-based buffer (TX-B; Nolan & Morgan, 1995), which was reported to extract protein from the 'cellular fraction', a single product of the appropriate size was seen. Treatment of the remaining cellular material with an SDS-based lysis

buffer (TX-B-SDS; Nolan & Morgan, 1995), reported to extract proteins from the 'nuclear fraction', liberated much more fusion protein. This 'nuclear fraction' contained fusion protein of the expected size as well as several smaller proteins. These may represent complete fusion protein in both glycosylated and unglycosylated form, in addition to degradation products. It appeared that with increased time in culture, a greater proportion of fusion protein became degraded. Degradation of $\alpha 11b$ fusion protein in insect cells has been attributed to the absence of dimer formation (Bennett *et al.*, 1993), but in the same study undimerised $\beta 3$ fusion protein was intact and readily secreted. The presence of two distinct fractions of $\beta 5$ fusion protein, in detergent soluble and insoluble compartments, was an additional complication. The detergent-insoluble fraction held the majority of the fusion protein, and could only be liberated on exposure to SDS lysis buffer. However this SDS-based lysis method caused denaturation of all proteins, thereby inactivating the GST-tag of the fusion protein which was to be used for protein purification. Therefore to enable subsequent purification it was necessary to collect fusion protein using detergent lysis, which gave low recovery of protein.

The presence of two fractions of fusion protein suggested that it was within distinct stores. The detergent-soluble store may have been exocytotic vesicles of fusion protein which were to be secreted but at levels at the limits of detection and too low for purification. The detergent-insoluble 'nuclear fraction' may contain fusion protein retained within the endoplasmic reticulum, which is a continuation of the nuclear envelope (Alberts *et al.*, 1989). This major store of protein was probably degraded in the absence of heterodimer formation. $\beta 5$ fusion protein breakdown products were detected by Western blotting using antibodies either to the amino-terminal GST-tag or the carboxy-terminal cytoplasmic tail, suggesting that degradation was not restricted to one end of the fusion protein. Breakdown products of a range of sizes were seen, suggesting that degradation was progressive.

The optimum time for fusion protein collection was determined by balancing the total amount of fusion protein expressed against the proportion degraded, and was shown to be day 4 of infection. The total amount of purified GST-tagged fusion protein collected from the 'cellular fraction' of the large scale culture in the present study was $\sim 40\mu\text{g}/100\text{ml}$ cell suspension, equivalent to $\sim 0.2\mu\text{g}$ per million cells. This compares favourably with Wippler *et al.* (1994) who reported a yield of $\sim 9\mu\text{g}/100\text{ml}$ cell suspension, equivalent to $\sim 0.02\mu\text{g}$ per million cells for $\beta 3$ fusion protein; this was collected from cell conditioned medium on day 4 of infection, with at least 20-fold less protein reported to be present in cell lysates. A yield of $\sim 90\mu\text{g}/100\text{ml}$ cell suspension has been reported for the $\alpha v\beta 3$ integrin dimer (Mehta *et al.*, 1998). Similar amounts were seen when a truncated form of the integrin was collected from cell-conditioned medium and when full length integrin was collected from cell lysates. This is equivalent to $\sim 4.5\mu\text{g}$ protein per million cells, both collected between day 3 and 4 of infection (Mehta *et al.*, 1998). Therefore using similar protocols the expression of related fusion proteins can be highly variable, and is possibly effected by the subunit-association of proteins which are not monomeric.

In the present study cells were infected with baculovirus at an MOI of 5 for large scale fusion protein production, as suggested by King & Posse (1994). The amount of virus (MOI) used for infection can have a significant effect on the amount of fusion protein expressed. It has been reported that reducing the MOI to 1 and collecting protein as early as day 2 of infection can provide a fusion protein yield of $2000\mu\text{g}/100\text{ml}$ cell suspension, equivalent to $20\mu\text{g}$ per million cells (Clark & Kulathila, 1997). However for this High FiveTM insect cells (Invitrogen) were used, which are reported to produce a better yield of fusion protein, as seen by Mehta *et al.* (1998). When SF9 cells were infected at an MOI of 1 to 2 with baculovirus encoding $\beta 3$ integrin (Wippler *et al.*, 1994) fusion protein expression was 10-fold lower than that seen in my work, indicating that fusion protein expression can be highly variable for reasons which are not easily explained. The amount of $\beta 5$ fusion protein expressed was only determined for the 'cellular fraction', at least the same amount again of fusion protein was trapped in detergent-insoluble stores although a proportion of this was degraded.

As I needed as much fusion protein as possible for raising antibodies, the GST-tag was not cleaved from the $\beta 5$ fusion protein prior to immunisation. It was expected that thrombin cleavage of GST and subsequent purification of $\beta 5$ would lead to loss of a significant amount of $\beta 5$ fusion protein. Therefore when anti- $\beta 5$ immune sera are collected they will need to be adsorbed against GST to remove any antibodies to this portion of the fusion protein. The GST tag was attached to the amino terminus of $\beta 5$, the effect of this on the protein folding of $\beta 5$ and the conformation of its ligand binding regions are not known. One indication of the integrity of $\beta 5$ fusion protein is that full length protein was readily detected by Western blotting using an antibody raised to a 30AA peptide of the cytoplasmic domain of $\beta 5$ (Smith *et al.*, 1990), demonstrating that $\beta 5$ fusion protein retains antigenicity.

From the range of sizes seen for GST-tagged fusion proteins, the average molecular weight of full length or ectodomain-only mouse $\beta 5$ fusion proteins (minus GST) was ~104kDa and ~92kDa respectively. However the additional band present on the silver gel in Figure 6.12b which may represent $\beta 5$ ectodomain without a GST tag is closer to 85kDa. Native human $\beta 5$ is ~90kDa (Hynes, 1987) with an ectodomain-only fragment expected to be ~80kDa. Therefore insect-cell expressed mouse integrin $\beta 5$ is comparable to human $\beta 5$ *in vivo*, although it appears 15% heavier. These differences can probably be attributed to glycosylation and species differences between human and mouse.

My work towards the present study had to end after the fusion protein was used for immunisation, due to the limits of time. However this work will continue in our laboratory. The immune sera generated will be first characterised by its ability to recognise purified $\beta 5$ fusion protein. It will also be used to demonstrate the distinct expression pattern of native $\beta 5$ that I have shown in endometrial and embryonic tissue. Sera will then be affinity purified to remove irrelevant antibodies (e.g. those to GST) and particularly to provide a reagent for use *in vitro*. For this purpose the IgG fraction of the affinity purified serum will be isolated for use in functional assays. The ability of antibodies to block integrin function could be determined using COS cells transfected with the mouse $\beta 5$

cDNA construct along with an αv construct; where attachment of these transfected COS cells to a substrate for $\alpha v\beta 5$ could be assayed.

Using the in vitro model of mouse implantation outlined in Chapter 5 the role of $\beta 5$ integrins in trophoblast attachment and outgrowth on a range of extracellular matrix substrates could be investigated. In addition a more complex in vitro model of implantation (Kimber *et al.*, 1993) can be used to determine the role of $\beta 5$ and other αv family integrin subunits in mediating attachment of mouse blastocysts to a polarised monolayer of mouse endometrial epithelium. Thus, the work which could follow on from this study would be towards revealing the role of the $\alpha v\beta 5$ integrin in the proposed integrin-mediated steps of the cell adhesion cascade which forms the basis of implantation.

CHAPTER 7

General discussion

In most species, including human and mouse, implantation requires direct adhesion of embryonic trophectoderm to the luminal epithelium of the endometrium, with subsequent penetration of the uterine stroma. This process is dependent on endometrial differentiation and embryo development to the blastocyst stage, and culminates in a cascade of cell adhesion events. The aim of the present study was to investigate the role of α_v integrins during implantation. The main findings were that:

- $\alpha_v\beta_5$ and $\alpha_v\beta_3$ are expressed at the apical surface of luminal uterine epithelial cells, and are present at the time of implantation;
- subunits α_v , β_1 , β_3 and β_5 are expressed at the trophectoderm of human and mouse blastocysts, and these become differentially distributed with outgrowth of trophoblasts on serum proteins;
- trophoblast outgrowth on FN or VN requires α_v while on LM β_1 is required, TSP does not support outgrowth.

Implantation involves many steps, each with different requirements for cell-cell and cell-matrix interactions. α_v integrins may have multiple roles during this temporal and spatially dynamic process.

Embryo attachment

Of the many cell adhesion-related genes which have been knocked out in mice very few have an implantation phenotype that prevents embryo attachment. During the course of this study, the results of an α_v integrin gene null mutation were reported; this showed no implantation phenotype (Hynes, 1996). Although embryos lacking $\alpha_v\beta_3$ and $\alpha_v\beta_5$ do implant this does not exclude the involvement of α_v integrins in implantation. As embryo attachment to the uterus is such a fundamental event in reproduction it is unlikely to be dependent on a single adhesive event. Indeed several adhesion systems are currently considered to mediate embryo attachment.

Uterine receptivity, which arises following appropriate hormonal priming, is essential for implantation. During the receptive phase, several α_v integrins are appropriately positioned on the uterine epithelium to mediate embryo attachment. However $\alpha_v\beta_5$ shows no obvious hormonal regulation, while $\alpha_v\beta_3$ shows subtle upregulation following progesterone treatment (Chapter 3). Alternative mechanisms may modulate their availability at the apical uterine epithelium. Upon receptivity, integrins may become exposed following downregulation of the epithelial mucins that mask integrins (Surveyor et al., 1995; Aplin, 1997). Receptivity-dependent, non-integrin events may be responsible for initiating the very first cell-cell adhesions at embryo attachment. Extended carbohydrate structures of the uterine epithelium which display glycan chains for which the blastocyst has receptors do appear to be steroidally regulated (Kimber et al., 1995). These may initiate embryo attachment, which this is then stabilised by integrin-ligand interactions. Such a model has previously been suggested by Kimber et al (1994b), which parallels embryo attachment to leukocyte-endothelial interaction at inflammation.

The apical location of several α_v integrins (including $\alpha_v\beta_3$, $\alpha_v\beta_5$) at the uterine epithelium indicates that these could be involved in embryo attachment (Chapter 3). α_v integrins do not usually mediate direct cell-cell binding. No cell-surface ligands have been identified for $\alpha_v\beta_5$ in mammalian cells, with PECAM-1 being the only cell-surface ligand identified for $\alpha_v\beta_3$ to date (Piali et al., 1995). α_v integrins are receptors for ECM components. They share the ability to bind the amino acid motif RGD present in many ECM molecules including FN, VN, TSP, OST. Injection of RGD peptides into the mouse uterine cavity reduces the rate of implantation (Bruce Lessey, personal communication). Similarly, injection of integrin-specific antibodies might allow identification of specific receptors involved in implantation (Chapter 5).

There are several potential mechanisms whereby α_v integrins expressed at both the surface of the embryonic trophectoderm (potentially $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$; Chapter 4) and that of the uterine luminal epithelium (predominantly $\alpha_v\beta_5$, with

$\alpha v\beta 3$; Chapter 3) could mediate embryo attachment. These would require an ECM component to act as a shared bridging ligand between αv integrins at the two cell surfaces. For this, the bridging ligand must possess at least two binding sites, one to be occupied by receptor at each epithelial surface.

Several potential bridging ligands exist. FN is displayed at the apical surface of the trophectoderm (Turpeenniemi-Hujanen et al., 1995); it forms a homodimer, and thus displays two RGD motifs. In addition both subunits have other cell-binding regions. TSP is also present at the apical surface of the trophectoderm (O'Shea et al., 1990); it forms a trimer, with potentially three RGD motifs. The present study demonstrated that blastocysts readily attach (then outgrow) on FN, but TSP was not able to support attachment in vitro indicating that this component is less likely to mediate embryo attachment in utero. OST can form polymers and can therefore display multiple RGD sites, this protein is upregulated around the time of implantation at the apical surface of the uterine luminal epithelium in human (Brown et al., 1992) and mouse (Craig & Denhardt, 1991). As this potential bridging ligand is regulated by progesterone this could determine uterine receptivity without the need for regulation of its receptors ($\alpha v\beta 3$, $\alpha v\beta 5$). Therefore an important avenue of further work (as discussed in Chapter 5) would be to examine the receptors required for trophoblast interaction with an OST substrate. Activated VN possesses an RGD recognition sequence and a high-affinity heparin binding domain (Felding-Haberman & Cheresh, 1993); however there is no current evidence for VN in the uterine cavity at implantation. A shared bridging ligand may be a soluble protein found within the uterine fluid, which is upregulated with the acquisition of uterine receptivity. A model could be envisaged whereby $\alpha v\beta 5$ on the uterine epithelium surface binds a soluble bridging ligand, which then becomes activated enabling binding of blastocyst receptors.

It is important to consider how integrins might interact with other adhesion systems implicated in implantation. One possible mechanism is binding of trophectoderm-associated HSPG (and EGF-R) to its transmembrane receptor

HB-EGF (Raab et al., 1996), which shows regulated expression at the uterine epithelial cell surface (Birdsall et al., 1996; Yoo et al., 1997), or to HIP, a newly identified heparin-binding protein (Rhode et al., 1996). It is not clear how HSPG is linked to the trophoctoderm surface, but it may involve association with another ECM component, which is in turn linked to an integrin on the trophoctoderm. Major candidates for this would be $\beta 1$ integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$); these are receptors for LM, which complexes with HSPG. However in $\beta 1$ -null mice, embryo attachment occurs apparently normally and it is only after trophoblast penetration of the uterine epithelium that implantation is disrupted (Fassler & Meyer, 1995; Stephens et al., 1995). In addition to LM, FN and TSP have heparin binding activity, suggesting a role for trophoctoderm αv integrins in mediating embryo attachment via HSPG-receptor binding; furthermore $\alpha v\beta 3$ could act directly by binding blastocyst HSPG core protein.

Although the mechanism of embryo attachment remains uncertain, it is likely that coordinated activity of multiple receptor-ligand interactions occur, encompassing several adhesion molecule systems. Also it is probable that initial adhesion must be strengthened. The present study has demonstrated that $\alpha v\beta 5$ (and $\alpha v\beta 3$) are in a position at the uterine epithelial surface to mediate such events, in both human and mouse. Upon integrin binding, activation to a high affinity state may be important. In this dynamic process, it may be that only firm adhesion of an embryo to the uterine epithelium, mediated by integrins, allows propagation of signals intracellularly so that the adhesion cascade of implantation can continue. Integrin upregulation and redistribution upon ligand binding may further reinforce the cell-cell adhesion. The microenvironment of an implantation site is also likely to regulate integrin activity, by effects on cation and integrin-ligand availability. These will stabilise integrins at the cell surface and/or modulate activity of other integrins.

Thus a possible model for embryo attachment in mouse, which is a development of the leukocyte-endothelial model (Kimber et al., 1994b), is as follows:

1. Loss of inhibitory mucins (e.g. MUC1) from the endometrium causes unmasking of integrins.
2. Initial embryo attachment occurs, mediated by steroidally regulated carbohydrates (e.g. LNF-1, HSPG).
3. Reinforcement of embryo attachment by bridging of endometrial and embryonic α_v integrins via a shared ligand (e.g. OST, FN), regulated by bridging ligand expression and/or integrin activation.
4. Integrin-induced signalling cascades - signals to the endometrium induce epithelial apoptosis, and signals to the trophoblast promote differentiation into invasive trophoblast.

Trophoblast interaction with the uterine epithelium basement membrane

Following embryo attachment, trophoblast cells must pass through the epithelial cell layer. In humans this probably occurs by intrusive penetration of the trophoblast through the maternal epithelium (Lindenberg, 1991), while in mouse this arises by displacement of the maternal epithelium facilitated by altered association between the basal surface of the uterine epithelium and its basement membrane (Blankenship & Given, 1995).

The present study identified intense immunolocalisation of β_5 at the basal surface of the luminal uterine epithelium in mouse, this was not seen in glands or in human uterine epithelium. α_v did not co-localise with β_5 at this location, suggesting pairing of β_5 with an alternative and as yet unidentified α subunit. This unusual pattern of β_5 expression suggests a special role in anchoring the mouse luminal epithelium to its basement membrane. Here β_5 may help maintain the integrity of the epithelial barrier until implantation occurs, at which time this integrin may be locally modulated by the implanting embryo. Accumulation of TN, a ligand for both α_v ($\alpha_v\beta_3$, $\alpha_v\beta_6$) and β_1 ($\alpha_8\beta_1$, $\alpha_9\beta_1$) integrins occurs below the luminal epithelium following embryo attachment in the mouse (Julian et al., 1994). This may disrupt the epithelial cell layer thereby facilitating epithelium displacement.

Once the trophoblasts have crossed the luminal uterine epithelium, they interact with the underlying basement membrane which mostly contains LM, COL IV and HSPG. It is at this stage that invasion ceases in mouse embryos lacking a functional $\beta 1$ gene (Fassler & Meyer, 1995; Stephens et al., 1995); indicating that $\beta 1$ integrins are essential for migration through this matrix probably by adhesion to LM and COL IV and that αv integrins cannot compensate here.

In the present study EHS matrix (containing LM-1 and COL IV) was not as efficient at supporting trophoblast outgrowth as VN or FN (Chapter 5); this might be because trophoblasts were not differentiated sufficiently to interact with these components. The observation that embryo attachment and/or outgrowth on EHS matrix may be delayed by 24 hours compared to VN or FN indicates that embryos lacked certain components required for interaction with EHS and that these were subsequently acquired, allowing outgrowth to proceed. Upregulation of integrins upon exposure to FN has previously been demonstrated in blastocysts. In utero, differentiation of trophoblasts may be required during embryo attachment and passage through the luminal epithelium to upregulate expression of receptors for basement membrane components. Indeed ligation of αv integrins has been shown to promote activity of $\beta 1$ integrins (Simon et al., 1997b).

Trophoblast invasion through the endometrial stroma

Once through the epithelial basement membrane, trophoblasts encounter the stromal ECM which is rich in ligands for both αv (FN, TSP, VN) and $\beta 1$ integrins (COLs, FN, LMs) as reviewed in section 1.8.1. The present study confirmed that FN, VN and LM support trophoblast cell outgrowth, and demonstrated that αv integrins are required for interaction with FN or VN, while LM requires $\beta 1$. It is possible that both αv and $\beta 1$ integrins are important in the stromal environment; however in the absence of $\beta 1$ gene function (Fassler & Meyer, 1995; Stephens et al., 1995) αv integrin cannot support trophoblast invasion through this matrix. This might be due to a lack of $\beta 1$ -derived signals which could promote

trophoblast differentiation, rather than the ability of trophoblast α_v integrins to bind stromal ECM components per se.

Trophoblast invasion is a complex process which must be strictly controlled so that it is sufficient to provide a blood supply to the fetus, but not excessive becoming akin to tumour metastasis. Therefore invasion-limiting factors must also be present deep in the stromal matrix. Alternative α_v integrin ligands can have differential effects on cell adhesion and migration. The present study showed that trophoblasts will not attach to or outgrow on TSP indicating that this might be such an invasion-limiting factor.

Gene knockouts of several ECM components have been produced in mice, without significant effect on implantation. Embryos lacking FN complete blastocyst formation and implant normally, but defects during embryogenesis lead to death before birth (George et al., 1993). Knockouts of VN, TSP or OST produce embryos that implant normally, are viable and have normal fertility (Zheng et al., 1995; Lawler et al., 1998; Liaw et al., 1998). Various LMs have been knocked out, producing embryos that implant normally but die before birth (Xu et al., 1994; Noakes et al., 1995; Miyagoe et al., 1997). However lack of LM γ_1 chain through gene mutation produces embryos which appear to implant normally until day 6.5 at which time implantation fails (Dr D Edgar, personal communication). The γ_1 chain of LM is found in several LMs, this null-mutation demonstrates the importance of LM in the stromal ECM during trophoblast invasion, and reflects findings in mice lacking β_1 integrin function (Fassler & Meyer, 1995; Stephens et al., 1995).

Consequences of integrin ligation at implantation

It seems that there are multiple stages during the dynamic process of implantation at which α_v integrins may be important, and also those where other mechanisms predominate (Figure 7.1). Common to all integrin-mediated steps is that they lead to two-way intracellular signalling cascades (reviewed in Chapter 1); 'inside-out signals' can modulate expression, ligand-binding and ligand-affinity

states of integrins. Intracellular signalling through integrins requires integrin clustering, and different integrin-ligand interactions induce alternate signalling cascades. The present study demonstrated that two patterns of clustering of trophoblast integrins could occur (Chapter 4). Focal adhesions were formed on the lower surface of cells by $\alpha v\beta 3$, whereas $\beta 1$ - and $\beta 5$ -containing integrins formed large clusters on the upper surface of trophoblasts. These may activate different signalling pathways by which cell function is modulated, and are probably crucial for implantation to continue. For example, integrin-induced signals may induce trophoblast differentiation into invasive trophoblast.

Integrin signalling pathways are intimately linked with those which mediate growth modulator and proteinase activity. LIF produced at uterine epithelium is essential for embryo attachment in mice (Stewart et al., 1992); this cytokine can enhance cell adhesion to FN by inducing upregulation of αv integrins (Heymann et al., 1996). Active proteinase (MMP-2) can bind $\alpha v\beta 3$ on the surface of invasive cells, which facilitates adhesion of cells to and degradation of COL (Brooks et al., 1996). Similar mechanisms are probably employed by trophoblasts as they penetrate the basement membrane of the uterine epithelium, and as they invade the stromal ECM.

With the production of new reagents, dissection of the roles of individual integrin pairs in implantation is possible. Hence the purification of $\beta 5$ integrin in this study (Chapter 6); production of antibodies against it (in progress) will allow investigation of the role of the $\alpha v\beta 5$ integrin in future work. Receptors that are essential during the attachment and subsequent invasion phases of implantation in utero will be elucidated as well as the signal cascades they generate. These intracellular signals, and the multitude of cellular functions they affect, hold the key to the dynamic and meticulously controlled process of embryo implantation.

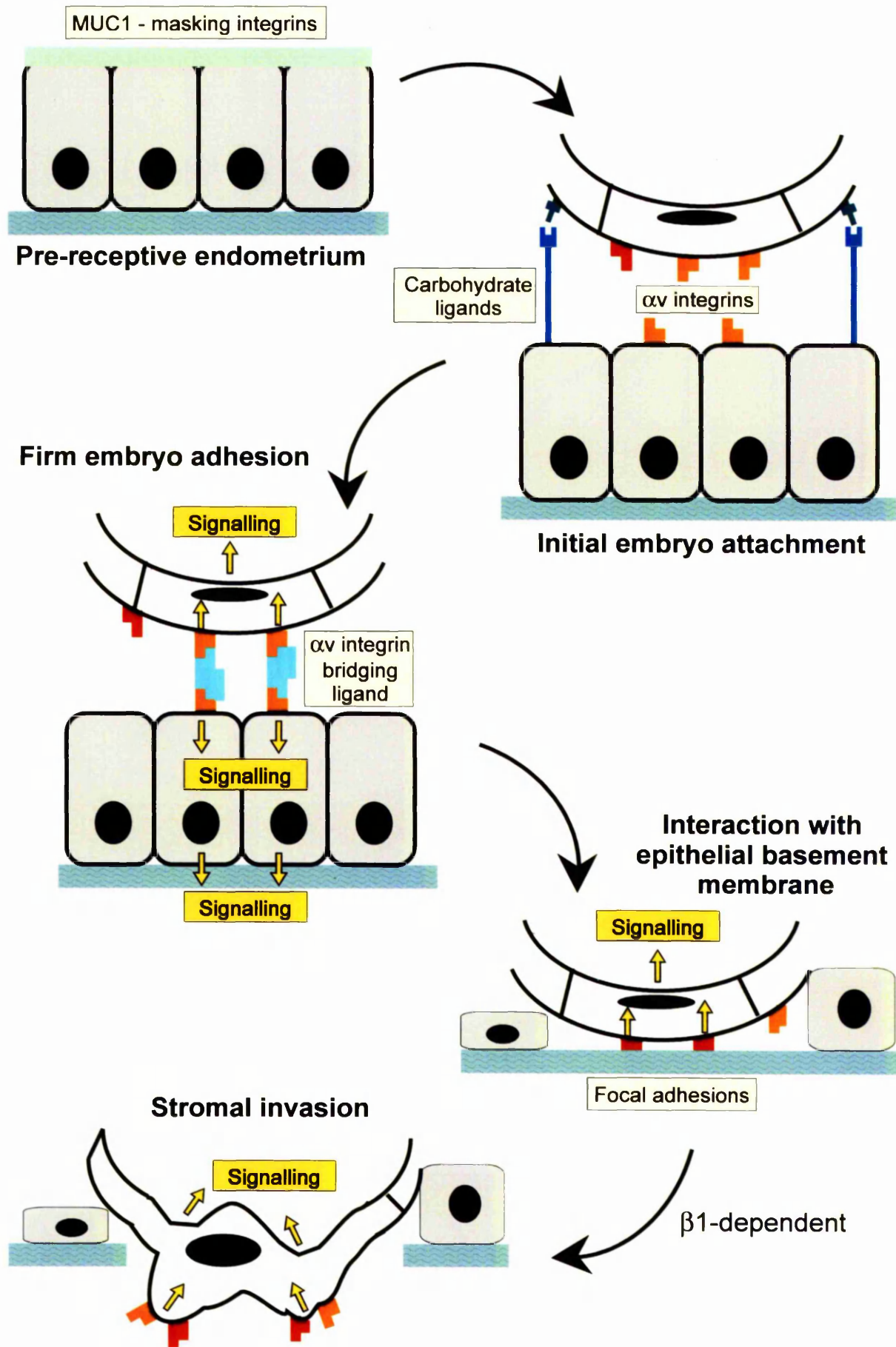


Figure 7.1 Embryo implantation: a model for the role of α_v integrins

REFERENCES

- Abbondanzo SJ, Cullinan EB, McIntyre K, Labow MA and Stewart CL (1996): Reproduction in mice lacking a functional type 1 IL-1 receptor. *Endocrinol.* 137, 3598-3601.
- Aboagye-Mathiesen G, Laugesen J, Zdravkovic M and Ebbesen P (1996): Isolation and characterization of human placental trophoblast subpopulations from first-trimester chorionic villi. *Clin. Diagn. Lab. Immunol.* 3, 14-22.
- Abrahamsohn PA and Zorn TMT (1993): Implantation and decidualization in rodents. *J. Exp. Zool.* 266, 603-628.
- Adams JC and Watt FM (1991): Expression of $\beta 1$, $\beta 3$, $\beta 4$ and $\beta 5$ integrins by human epidermal keratinocytes and non-differentiating keratinocytes. *J. Cell Biol.* 115, 829-841.
- Agrez MV, Bates RC, Mitchell D, Wilson N, Ferguson N, Anselme P and Sheppard D (1996): Multiplicity of fibronectin-binding αv integrin receptors in colorectal cancer. *Br. J. Cancer.* 73, 887-892.
- Agrez MV, Chen A, Cone RI, Pytela R and Sheppard D (1994): The $\alpha v \beta 6$ integrin promotes proliferation of colon carcinoma cells through a unique region of the $\beta 6$ cytoplasmic tails. *J. Cell Biol.* 127, 547-556.
- Akiyama SK, Yamada SS, Yamada KM and LaFemine SE (1994): Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J. Biol. Chem.* 269, 15961-15964.
- Albeda SM and Buck CA (1990): Integrins and other cell adhesion molecules. *FASEB J.* 4, 2868-2880.
- Albers A, Thie M, Hohn HP and Denker HW (1995): Differential expression and localization of integrins and CD44 in the membrane domains of human uterine epithelial cells during the menstrual cycle. *Acta. Anat.* 153, 12-19.
- Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD (eds.) (1989): In, *Molecular biology of the cell*. Garland Publishing, New York.
- Aplin JD (1997): Adhesion molecules in implantation. *Rev. Reprod.* 2, 84-93.
- Aplin JD (1991): Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. *J. Cell Sci.* 99, 681-692.
- Aplin JD and Glasser SR (1994): In, *Endocrinology of embryo-endometrium interactions* (ed. Glasser SR *et al.*). Plenum Press, New York.
- Aplin JD, Charlton AK and Ayad S (1988): An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res.* 253, 231-240.
- Aplin JD, Hey NA and Graham A (1998): Human endometrium MUC1 carries keratan sulfate: characteristic glycoforms in the luminal epithelium at receptivity. *Glycobiology.* 8, 269-276.

- Aplin JD, Mylona P, Kielty CM, Ball S, Williams JDL, Church HJ and Jones CJP (1995): In, *Molecular and cellular aspects of periimplantation processes* (ed. Dey SK); Sero symposia. Springer-Verlag, NY.
- Aplin JD, Spanswick C, Behzad F, Kimber SJ and Vicovac LJ (1996): Integrins $\beta 5$, $\beta 3$ and αv are apically distributed in endometrial epithelium. *Molec. Hum. Reprod.* 2, 527-534.
- Arici A, Engin O, Attar E and Olive DL (1995): Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J. Clin. Endocrinol. Metab.* 80, 1908-1915.
- Armant DR (1991): Cell interaction with laminin and its proteolytic fragments during outgrowth of mouse primary trophoblast cells. *Biol. Reprod.* 45, 664-672.
- Armant DR and Kameda S (1994): Mouse trophoblast cell invasion of extracellular matrix purified from endometrial tissue: A model for peri-implantation development. *J. Exp. Zool.* 269, 146-156.
- Armant DR, Kaplan HA and Lennarz WJ (1986a): Fibronectin and laminin promote in vitro attachment and outgrowth of mouse blastocysts. *Devel Biol.* 116, 519-523.
- Armant DR, Kaplan HA, Mover H and Lennarz WJ (1986b): The effects of hexapeptides on attachment and outgrowth of mouse blastocysts cultured in vitro: Evidence for the involvement of the cell recognition tripeptide Arg-Gly-Asp. *Proc. Natl. Acad. Sci. USA.* 83, 6751-6755.
- Aznavoorian S, Stracke ML, Parsons J, McClanahan J, Liotta LA (1996): Integrin $\alpha v \beta 3$ mediates chemotactic and haptotactic motility in human melanoma cells through different signalling pathways. *J. Biol. Chem.* 271, 3247-3254.
- Babiarz B, Romagnano L, Afonso S and Kurila G (1996): Localization and expression of fibronectin during mouse decidualization in vitro: mechanisms of cell:matrix interactions. *Dev. Dyn.* 206, 330-342.
- Bajt ML and Loftus JC (1994): Mutation of a ligand binding domain of $\beta 3$ integrin. *J. Biol. Chem.* 269, 20913-20919.
- Baker J, Liu J-P, Robertson E and Efstatidis A (1993): Role of insulin-like growth factors in embryonic and postnatal growth. *Cell.* 75, 73-82.
- Balzac F, Retta SF, Albini A, Melchiorri A, Kotliansky VE, Geuna M, Silengo L and Tarone G (1994): Expression of $\beta 1 B$ integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. *J. Cell Biol.* 127, 557-565.
- Barnea ER, Feldman D, Kaplan M and Morrish DW (1990): The dual effect of epidermal growth factor upon human chorionic gonadotrophin secretion by the first trimester placenta in vitro. *J. Clin. Endocrinol. Metab.* 71, 923-928.
- Barondes SH, Cooper DN, Gitt MA and Leffler H (1994): Galectins. Structure and function of a large family of animal lectins. *J. Biol. Chem.* 269, 20807-20810.

- Bazzoni G, Shih DT, Buck CA and Hemler ME (1995): Monoclonal antibody 9EG7 defines a novel beta 1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. 270, 25570-25577.
- Beardsley TR, Pierschbacher M, Wetzel GD and Hays EF (1983): Induction of T-cell maturation by a cloned line of thymic epithelium (TEPI). *Proc. Natl. Acad. Sci. USA*. 80, 6005-6009.
- Beato M (1989): Gene regulation by steroid hormones. *Cell*. 56, 335-344.
- Behzad F, Seif MW, Campbell S and Aplin JD (1994): Expression of two isoforms of CD44 in human endometrium. *Biol. Reprod.* 51, 739-747.
- Bennett JS and Vilaire G (1979): Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest.* 64, 1393-1401.
- Bennet JS, Kolodziej MA, Vilaire G and Poncz M (1993): Determinants of the intracellular fate of truncated forms of the platelet glycoproteins IIb and IIIa. *J. Biol. Chem.* 268, 3580-3585.
- Bergeron C, Ferenczy A and Shyamala G (1988): Distribution of estrogen receptors in various cell types of normal, hyperplastic, and neoplastic human endometrial tissues. *Lab. Invest.* 58, 338-345.
- Bergh PA and Navot D (1992): The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertil. Steril.* 58, 537-542.
- Bergman M, Joukov V, Virtanen I and Alitalo K (1995): Overexpressed csk tyrosine kinase is localized in focal adhesions, causes reorganisation of $\alpha v \beta 5$ integrin, and interferes with HeLa cell spreading. *Molec. Cell. Biol.* 15, 711-722.
- Bhatt H, Brunet LJ and Stewart CL (1991): Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natl. Acad. Sci. USA* 88, 11408-11412.
- Bhattacharya S, Fu C, Bhattacharya J and Greenberg S (1995): Soluble ligands of the $\alpha v \beta 3$ integrin mediate enhanced tyrosine phosphorylation of multiple proteins in adherent bovine pulmonary artery endothelial cells. *J. Biol. Chem.* 270, 16781-16787.
- Biggers JD, Lawitts JA and Lechene CP (1993). The protective action of betaine on the deleterious effects of NaCl on pre-implantation mouse embryos in vitro. *Mol. Reprod. Dev.* 34, 380-390.
- Bigler D, Chen M, Waters S and White JM (1997): A model for sperm-egg binding and fusion based on ADAMs and integrins. *Trends Cell Biol.* 7, 220-225.
- Birdsall MA, Hopkisson JF, Grant KE, Barlow DH and Mardon HJ (1996): Expression of heparin-binding epidermal growth factor messenger RNA in the human endometrium. *Mol. Hum. Reprod.* 2, 31-34.

- Bischof P, Redard M, Gindre P, Vassilakos P and Campana A (1993): Localization of alpha 2, alpha 5 and alpha 6 integrin subunits in human endometrium, decidua and trophoblast. *Eur. J. Obs. Gyn. Reprod. Biol.* 51, 217-226.
- Blankenship TN and Given RL (1995): Loss of laminin and type IV collagen in uterine luminal epithelial basement membranes during blastocyst implantation in the mouse. *Anat. Rec.* 243, 27-36.
- Blystone SD, Graham IL, Lindberg FP and Brown EJ (1994): Integrin $\alpha v \beta 3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor. *J. Cell Biol.* 127, 1129-1137.
- Blystone SD, Lindberg FP, Williams MP, McHugh KP and Brown EJ (1996): Inducible tyrosine phosphorylation of the beta3 integrin requires the alphav integrin cytoplasmic tail. *J. Biol. Chem.* 271, 31458-31462.
- Borland RM and Tasca RJ (1974): Activation of a Na⁺ -dependent amino acid transport system in preimplantation mouse embryos. *Devel. Biol.* 36, 169-182.
- Bornstein P (1992): Thrombospondins: Structure and regulation of expression. *FASEB J.* 6, 3290-3299.
- Bouchard P, Marraoui J, Massai MR, Medalie DA, de Ziegler D, Perrot-Appianat M, Frydman R and Bergeron C (1991): Immunocytochemical localization of oestrodial and progesterone receptors in human endometrium: a tool to assess endometrial maturation. *Bailliere's Clin. Obstet. Gynaecol.* 5, 107-115.
- Bowman P and McLaren A (1970): Cleavage rate of mouse embryos in vivo and in vitro. *J. Embryol. Exp. Morphol.* 24, 203-207.
- Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Braga VM and Gendler SJ (1993): Modulation of Muc-1 mucin in the mouse uterus during the estrus cycle, early pregnancy and placentation.
- Brakebush C, Hirsch E, Potocnik A and Fassler R (1997): Genetic analysis of $\beta 1$ function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. *J. Cell Sci.* 110, 2895-2904.
- Braude P, Bolton V and Moore S (1988): Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature.* 332, 459-461.
- Breuss JM, Gallo J, DeLisser HM, Klimanskaya IV, Folkesson HG, Pittet JF, Nishimura SL, Aldape K, Landers DV, Carpenter W, Gillett N, Sheppard D, Matthay MA, Albeda SM, Kramer RH and Pytela R (1995): Expression of the $\beta 6$ integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodelling. *J. Cell Sci.* 108, 2241-2251.
- Breuss JM, Gillett N, Lu L, Sheppard D and Pytela R (1993): Restricted distribution of integrin $\beta 6$ mRNA in primate epithelial tissues. *J. Histo. Cytol.* 41, 1521-1527.

- Bridges JE, Prentice A, Roche W, Englefield P and Thomas EJ (1994): Expression of integrin adhesion molecules in endometrium and endometriosis. *Br. J. Obs. Gyn.* 101, 696-700.
- Bronson RA and Fusi FM (1996): Integrins and human reproduction. *Mol. Hum. Reprod.* 2, 153-168.
- Brooks PC, Klemke RL, Schon S, Lewis JM, Schwartz MA and Cheresch DA (1997): Insulin-like growth factor receptor cooperates with integrin $\alpha v \beta 5$ to promote tumour cell dissemination in vivo. *J. Clin. Invest.* 99, 1390-1398.
- Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP and Cheresch DA (1996): Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha v \beta 3$. *Cell.* 85, 683-693.
- Brown EJ (1997): Adhesive interactions in the immune system. *Trends Cell Biol.* 7, 289-295.
- Brown LF, Berse B, Van de Water L, Papadopoulos-Sergiou A, Perruzzi CA, Manseau EJ, Dvorak HF and Senger DR (1992): Expression and distribution of osteopontin in human tissues: Widespread association with luminal epithelial surfaces. *Molec. Biol. Cell* 3, 1169-1180.
- Buckley CD, Doyonnas R, Newton JP, Blystone SD, Brown EJ, Watt SM and Simmons DL (1996): Identification of $\alpha v \beta 3$ as a heterotypic ligand for CD31/PECAM-1. *J. Cell Sci.* 109, 437-445.
- Burrows TD, King A and Loke YW (1996): Trophoblast migration during human placental implantation. *Hum. Reprod. Update.* 2, 307-321.
- Burrows TD, King A and Loke YW (1993): Expression of integrins by human trophoblast and differential adhesion to laminin and fibronectin. *Hum. Reprod.* 8, 475-484.
- Burrows TD, King A, Smith SK and Loke YW (1997): In, *Trophoblast research 10* (ed. Foidart JM, Aplin JD, Kaufmann P and Schaaps J-P). University of Rochester press, NY.
- Burrows TD, King A, Smith SK and Loke YW (1995): Human trophoblast adhesion to matrix proteins: inhibition and signal transduction. *Molec. Hum. Reprod.* 1, 2489-2500.
- Campbell S, Swann HR, Aplin JD, Seif MW, Kimber SJ and Elstein M (1995a): CD44 is expressed throughout pre-implantation human embryo development. *Hum. Reprod.* 10, 425-430.
- Campbell S, Swann HR, Seif MW, Kimber SJ and Aplin JD (1995b): Cell adhesion molecules on the oocyte and preimplantation human embryo. *Hum. Reprod.* 10, 1571-1578.
- Carson DD, Farrar JD, Laidlaw J and Wright DA (1990a): Selective activation of the N-glycosylation apparatus in uteri by estrogen. *J. Biol. Chem.* 265, 2947-2955.

- Carson DD, Jacobs, AL, Julian J, Rohde LH and Valdizan MC (1992a): Glycoconjugates as positive and negative modulators of embryo implantation. *Reprod. Fertil. Devel.* 4, 271-274.
- Carson DD, Julian J and Jacobs, AL (1992b): Uterine stromal cell chondroitin sulfate proteoglycans bind to collagen type I and inhibit embryo outgrowth in vitro. *Devel. Biol.* 149, 307-316.
- Carson DD, Rohde LH and Surveyor G (1994): Cell surface glycoconjugates as modulators of embryo attachment to uterine epithelial cells. *Int. J. Biochem.* 26, 1269-1277.
- Carson DD, Tang JP and Gay S (1988): Collagens support embryo attachment and outgrowth in vitro: Effects of the Arg-Gly-Asp sequence. *Devel. Biol.* 127, 368-375.
- Carson DD, Tang JP and Julian J (1993): Heparan sulfate proteoglycan (perlecan) expression by mouse embryos during acquisition of attachment competence. *Devel. Biol.* 155, 97-106.
- Carson DD, Wilson OF and Dutt A (1990b): In: *Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. Trophoblast research 4.* (ed. Denker HW & Aplin JD). Plenum Press, New York.
- Castelbaum AJ, Sawin SW, Bellardo LJ and Lessey BA (1995): Endometrial integrin expression in women exposed to diethylstilbestrol in utero. *Fertil. Steril.* 63, 1217-1221.
- Chakraborty I, Das SK and Dey SK (1995): Differentiation of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. *J. Endocrinol.* 147, 339-352.
- Charnock-Jones DS, Sharkey AM, Fenwick P and Smith SK (1994): Leukemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J. Reprod. Fertil.* 101, 421-426.
- Charnock-Jones DS, Sharkey AM, Rajput-Williams J, Burch D, Schfield JP, Fountain SA, Boocock CA and Smith SK (1993): Identification and localization of alternatively spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. *Biol. Reprod.* 48, 1120-1128.
- Chavez DJ (1990): In: *Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. Trophoblast research 4.* (ed. Denker HW & Aplin JD). Plenum Press, New York.
- Chavez DJ (1984): Embryology of the mouse from ovulation through peri-implantation stages in vitro. *Scan. Elec. Microscop.* 2, 729-735.
- Chellaiah M, Fitzgerald C, Filardo EJ, Cheresh DA and Hruska KA (1996): Osteopontin activation of c-src in human melanoma cells requires the cytoplasmic domain of the integrin alpha v-subunit. *Endocrinol.* 137, 2432-2440.

- Chen J, Maeda T, Sekiguchi K and Sheppard D (1996): Distinct structural requirements for interaction of the integrins $\alpha 5 \beta 1$, $\alpha v \beta 5$, and $\alpha v \beta 6$ with the central cell binding domain in fibronectin. *Cell Adhes. Commun.* 4, 237-250.
- Cheresh DA (1987): Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84, 6471-6475.
- Choudhuri R and Wood GW (1993): Production of interleukin-1, interleukin-6, and tumour necrosis factor alpha in the uterus of pseudopregnant mice. *Biol. Reprod.* 49, 596-603.
- Church HJ, Richards AJ and Aplin JD (1997): In, *Trophoblast research 10* (ed. Foidart JM, Aplin JD, Kaufmann P and Schaaps J-P). University of Rochester press, NY.
- Church HJ, Vicovac Lj, Williams JDL, Hey NA and Aplin JD (1996): Laminins 2 and 4 are expressed by human decidual cells. *Lab. Invest.* 74, 21-32.
- Clark K and Kulathila R (1997): An MOI time course improved protein yield in baculovirus. *Expressions (product application notes, Invitrogen).* 4 (4), 13.
- Classen-Linke I and Denker HW (1990): In: *Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. Trophoblast research 4.* (ed. Denker HW & Aplin JD). Plenum Press, New York.
- Collins JE and Fleming TP (1995): Epithelial differentiation in the mouse preimplantation embryo: making adhesive cell contacts for the first time. *TIBS.* 20, 307-312.
- Cooper AR and MacQueen HA (1983): Subunits of laminin are differentially synthesized in mouse eggs and early embryos. *Devel. Biol.* 96, 467-471.
- Coppens MT, De Boever JG, Dhont MA, Serreyn RF, Vandekerckhove DA and Roels HJ (1993): Topographical distribution of oestrogen and progesterone receptors in the human endometrium and Fallopian tube. *Histochem.* 99, 127-131.
- Corless CL, Mendoza A, Collins T, Lawler J (1992): Colocalisation of thrombospondin and syndecan during murine development. *Dev. Dyn.* 193, 346-358.
- Cowell TP (1969): Implantation and the development of mouse eggs transferred to the uteri of non-progestational mice. *J. Reprod. Fertil.* 19, 239-245.
- Craig WS, Cheng S, Mullen DG, Blevitt J and Pierschbacher MD (1995): Concept and progress in the development of RGD-containing peptide pharmaceuticals. *Biopolymers.* 37, 157-175.
- Craig AM and Denhardt DT (1991): The murine gene encoding secreted phosphoprotein 1 (osteopontin): Promoter structure, activity, and induction in vivo by oestrogen and progesterone. *Gene* 100, 163-171.
- Cross JC, Werb Z and Fisher SJ (1994): Implantation and the placenta: key pieces of the developmental puzzle. *Science.* 266, 1508-1518.

- Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA and Stewart CL (1996): Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proc. Natl. Acad. Sci. USA* 93, 3115-3120.
- Daiter E, Omigbodun A, Wang S, Walinsky D, Strauss (III) JF, Hoyer JR and Coutifaris C (1996): Cell differentiation and endogenous cyclic adenosine 3'-5'-monophosphate regulate osteopontin expression in human trophoblasts. *Endocrin.* 137, 1785-1790.
- Dalton SL, Scharf E, Briesewitz R, Marcantonio EE and Assoian RK (1995): Cell adhesion to extracellular matrix regulates the life cycle of integrins. *Molec. Biol. Cell.* 6, 1781-1791.
- Damsky CH, Fitzgerald ML and Fisher SJ (1992): Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J. Clin. Invest.* 89, 210-222.
- Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK and Fisher SJ (1994): Integrin switching regulates normal trophoblast invasion. *Devel.* 120, 3657-3666.
- Damsky CH, Moursi A, Zhou Y, Fisher SJ and Globus RK (1997): The solid state environment orchestrates embryonic development and tissue remodeling. *Kidney Int.* 51, 1427-1433.
- Damsky C, Sutherland A and Fisher S (1993): Extracellular matrix 5: Adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *FASEB J.* 7, 1320-1329.
- Dardik A, Smith RM and Schultz RM (1992): Colocalization of transforming growth factor- α and a functional epidermal growth factor receptor (EGFR) to the inner cell mass and preferential localization of the EGFR on the basolateral surface of the trophectoderm in the mouse blastocyst. *Devel. Biol.* 154, 396-409.
- Das SK, Chakraborty I, Paria BC, Wang X-N, Plowman G and Dey SK (1995): Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. *Molec. Endocrin.* 9, 691-705.
- Das SK, Wang X-N, Paria BC, Damm D, Abraham JA, Klagsbrun M, Andrews GK and Dey SK (1994): Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Devel.* 120, 1071-1083.
- De M, Sanford TR and Wood GW (1993): Expression of interleukin 1, interleukin 6 and tumour necrosis factor α in mouse uterus during the peri-implantation period of pregnancy. *J. Reprod. Fertil.* 97, 83-89.
- De Nichilo MO and Burns GF (1993): Granulocyte-macrophage colony-stimulating factors differentially regulate αv integrin expression on cultured human macrophages. *Proc. Natl. Acad. Sci. USA.* 90, 2517-2521.

- De Nichilo MO and Yamada KM (1996): Integrin $\alpha v \beta 5$ -dependent serine phosphorylation of paxillin in cultured human macrophages adherent to vitronectin. *J. Biol. Chem.* 271, 11016-11022.
- Dedhar S and Hannigan GE (1996): Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell Biol.* 8, 657-669.
- Delannet M, Martin F, Bossy B, Cheresh DA, Reichardt LF and Duband J-L (1994): Specific roles of the $\alpha v \beta 1$, $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins in avian neural crest cell adhesion and migration on vitronectin. *Devel.* 120, 2887-2702.
- Delcommenne M and Streuli CH (1995): Control of integrin expression by the extracellular matrix. *J. Biol. Chem.* 270, 26794-26801.
- Denhardt DT and Guo X (1993): Osteopontin: a protein with diverse functions. *FASEB J.* 7, 1475-1482.
- Denker HW (1994): In: *Endocrinology of embryo-endometrium interactions* (ed. Glasser SR, Mulholland J & Psychoyos A). Plenum Press, New York.
- Denker HW (1990): In: *Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. Trophoblast research 4.* (ed. Denker HW & Aplin JD). Plenum Press, New York.
- Diamond MS and Springer TA (1994): The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 4, 506-517.
- Doerr ME and Jones JI (1996): The roles of integrins and extracellular matrix proteins in the insulin-like growth factor I-stimulated chemotaxis of human breast cancer cells. *J. Biol. Chem.* 271, 2443-2447.
- Dou Q, Williams RS and Chegini N (1997): Evidence that human endometrium throughout the menstrual cycle expresses messenger RNA for integrins. *J. Soc. Gynecol. Invest.* 4, A215.
- D'Souza SE, Ginsberg MH, Burke TA, Lam SCT and Plow EF (1988): Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 242, 91-93.
- Du X, Gu M, Weiel JW and Nagaswami C (1993): Long range propagation of conformational changes in integrin $\alpha L \beta 3$. *J. Biol. Chem.* 268, 23087-23092.
- Dziadek M and Timpl R (1985): Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. *Devel. Biol.* 111, 372-382.
- Evans JP, Schultz RM and Kopf GS (1995): Identification and localization of integrin subunits in oocytes and eggs of the mouse. *Mol. Reprod. Devel.* 40, 211-220.
- Farach MC, Tang JP, Decker GL and Carson DD (1988): Differential effects of p-nitrophenyl-D-xylosides on mouse blastocysts and uterine epithelial cells. *Biol. Reprod.* 39, 443-455.

- Farach MC, Tang JP, Decker GL and Carson DD (1987): Heparin/heparan sulfate is involved in attachment and spreading of mouse embryos in vitro. *Devel. Biol.* 123, 401-410.
- Farrar JD and Carson DD (1992): Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. *Biol. Repro.* 46, 1095-1108.
- Fassler R and Meyer M (1995): Consequences of lack of $\beta 1$ integrin gene expression in mice. *Genes & Devel.* 9, 1896-1908.
- Fassler R, Georges-Labouesse E and Hirsch E (1996): Genetic analysis of integrin function in mice. *Curr. Op. Cell Biol.* 8, 641-646.
- Fassler R, Pfaff M, Murphy J, Noegel AA, Johansson S, Timpl R and Albrecht R (1995): Lack of $\beta 1$ integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J. Cell Biol.* 128, 979-988.
- Fazleabas AT, Bell SC, Fleming S, Sun J and Lessey BA (1997): Distribution of integrins and the extracellular matrix proteins in the baboon endometrium during the menstrual cycle and early pregnancy. *Biol. Reprod.* 56, 348-356.
- Feinberg RF, Kliman HJ and Lockwood CJ (1991): Is oncofetal fibronectin a trophoblast glue for human implantation? *Am. J. Pathol.* 138, 537-543.
- Felding-Habermann B and Cheresh DA (1993): Vitronectin and its receptors. *Curr. Opin. Cell Biol.* 5, 864-868.
- Fenderson BA, Holmes EH, Fukushi Y and Hakomori S (1986): Coordinate expression of X and Y haptens during murine embryogenesis. *Devel. Biol.* 114, 12-21.
- Filardo EJ and Cheresh DA (1994): A β turn in the cytoplasmic tail of the integrin αv subunit influences conformation and ligand binding of $\alpha v \beta 3$. *J. Biol. Chem.* 269, 4641-4647.
- Filardo EJ, Brooks PC, Deming SL, Damsky C and Cheresh DA (1995): Requirement of the NPXY motif in the integrin $\beta 3$ subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. *J. Biol. Chem.* 270, 441-450.
- Filardo EJ, Demming SL and Cheresh DA (1996): Regulation of cell migration by the integrin β subunit ectodomain. *J. Cell Sci.* 109, 1615-1622.
- Finn CA (1977): In, *Biology of the uterus* (ed. Wynn RM). Plenum Press, New York.
- Finn CA and Martin L (1970): The role of the oestrogen secreted before oestrus in the preparation of the uterus for implantation in the mouse. *J. Endocr.* 47, 431-438.
- Finn CA and Martin L (1969): Hormone secretion during early pregnancy in the mouse. *J. Endocrinol.* 45, 57-65.

- Finn CA and Martin L (1967): Patterns of cell division in the mouse uterus during early pregnancy. *J. Endocrinol.* 39, 593-597.
- Fischer B, Jung T, Hegele-Hartung C and Beier HM (1990): Development of preimplantation rabbit embryos in uterine flushing-supplemented culture media. *Mol. Reprod. Dev.* 27, 216-223.
- Fleming TP, Butler L, Lei X, Collins J, Javed Q, Sheth B, Stoddart N, Wild A and Hay M (1994): Molecular maturation of cell adhesion systems during mouse early development. *Histochem.* 101, 1-7.
- Fleming TP, Garrod DR and Elsmore AJ (1991): Desmosome biogenesis in the mouse preimplantation embryo. *Devel.* 112, 527-539.
- Fleming TP, Javed Q and Hay M (1992): Epithelial differentiation and intercellular junction formation in the mouse early embryo. *Devel.* 1992 Supp., 105-112.
- Fleming TP, Javed Q, Collins J and Hay M (1993): Biogenesis of structural intercellular junctions during cleavage in the mouse embryo. *J. Cell Sci. Supp.* 17, 119-125.
- Frank HG, Malekzadeh F, Kertschanska S, Crescimanno C, Castelucci M, Lang I, Desoye G and Kaufmann P (1994): Immunohistochemistry of two different types of placental fibrinoid. *Acta Anat.* 150, 55-68.
- Fukuda MN, Sato T, Nakayama J, Klier G, Mikami M, Aoki D and Nozawa S (1995): Trophinin and tasin, a novel cell adhesion molecule complex with potential involvement in embryo implantation. *Gene. Devel.* 9, 1199-1210.
- Fusi FM, Tamburini C, Mangili F, Montesano M, Ferrari A and Bronson RA (1996): The expression of alpha v, alpha 5, beta 1 and beta 3 integrin chains on ejaculated human spermatozoa varies with their functional state. *Mol. Hum. Reprod.* 2, 169-175.
- Fusi FM, Vignali VM, Busacca M and Bronson RA (1992): Evidence for the presence of an integrin cell adhesion receptor on the oolemma of unfertilized human oocytes. *Mol. Reprod. Devel.* 31, 215-222.
- George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H and Hynes RO (1993): Defects in mesoderm, neural tube and vasculature development in mouse embryos lacking fibronectin. *Devel.* 119, 1079-1091.
- Giancotti FG (1997): Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.* 9, 691-700.
- Giudice LC and Saleh W (1995): Growth factors in reproduction. *Trends Endocrinol. Metab.* 6, 60-69.
- Giudice LC (1994): Growth factors and growth modulators in human endometrium: their potential relevance to reproductive medicine. *Fertil. Steril.* 61, 1-17.
- Glasser SR and Mulholland J (1993): Receptivity is a polarity dependant special function of hormonally regulated uterine epithelial cells. *Microscop. Res. Tech.* 25, 106-120.

- Glasser SR, Julian J, Decker GL, Tang JP and Carson DD (1988): Development of morphological and functional polarity in primary cultures of immature rat uterine epithelial cells. *J. Cell Biol.* 107, 2409-2423.
- Glasser SR, Lampelo S, Munir MI and Julian J (1987): Expression of desmin, laminin and fibronectin during in situ differentiation (decidualization) of rat uterine stromal cells. *Differentiation* 35, 132-142.
- Glenister TW (1961): Organ culture as a new method for studying the implantation of mammalian blastocysts. *Proc. R. Soc. Lond. (Biol.)* 154, 428-431.
- Gold LI, Saxena B, Mittal KR, Marmor M, Goswami S, Nactigal L, Korc M and Demopoulos RI (1994): Increased expression of transforming growth factor beta isoforms and basic fibroblast growth factor in complex hyperplasia and adenocarcinoma of the endometrium: evidence for paracrine and autocrine action. *Cancer Res.* 54, 2347-2358.
- Gonzales DS, Jones JM, Pinyopummintr T, Carnevale EM, Ginther OJ, Shapiro SS and Bavister BD (1996): Trophoctoderm projections: apotential means for locomotion, attachment and implantation of bovine, equine and human blastocysts. *Hum. Reprod.* 11, 2739-2745.
- Graham RA, Li TC, Cooke ID and Aplin JD (1994): Keratan sulphate as a secretory product of human endometrium: cyclic expression in normal women. *Hum. Reprod.* 9, 926-930.
- Grey K, Eitzman B, Raszmahn K, Steed T, Geboff A, McLachlan L and Bidwell M (1995): Coordinate regulation by diethylstilbestrol of the platelet-derived growth factor-A (PDGF-A) and -B chains and the PDGF receptor α - and β - subunits in the mouse uterus and vagina: potential mediators of estrogen action. *Endocrinol.* 136, 2325-2340.
- Grosskinsky CM, Yowell CW, Sun J, Parise LV and Lessey BA (1996): Modulation of integrin expression in endometrial stromal cells in vitro. *J. Clin. Endocrinol. Metab.* 81, 2047-2054.
- Haas TA and Plow EF (1994): Integrin-ligand interactions: a year in review. *Curr. Opin. Cell Biol.* 6, 656-662.
- Haimovici F and Anderson DJ (1993): Effects of growth factors and growth factor-extracellular matrix interactions on mouse trophoblast outgrowth in vitro. *Biol. Reprod.* 49, 124-130.
- Haralson MA and Hassell JR (1995): In, *Extracellular matrix: a practical approach* (ed. Haralson MA and Hassell JR). Oxford University Press, New York.
- Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zang X, Edwards DR and Schultz GA (1995): Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. *Devel.* 121, 1005-1014.
- Hay E (ed.) (1991): *Cell biology of the extracellular matrix*. Plenum Press, New York.

- Hayman EG, Piersbacher MD, Suzuki S and Ruoslahti E (1985): Vitronectin - a major cell attachment promoting protein in fetal bovine serum. *Exp. Cell Res.* 160, 245-258.
- Healy JM, Murayama O, Maeda T, Yoshino S and Kikuchi M (1995): Peptide ligands for integrin α v β 3 selected from random display libraries. *Biochem.* 34, 3948-3955.
- Hedlund K and Nilsson O (1971): Hormonal requirements for uterine attachment reaction and blastocyst implantation in the mouse, hamster and guinea pig. *J. Reprod. Fertil.* 47, 59-62.
- Hedman H and Lundgren E (1996): Regulation of α -4 integrin avidity in human B cells - requirement for dephosphorylation events for high avidity VCAM-1 binding. *J. Immunol.* 44, 239-242.
- Hertig AT, Rock J and Adams EC (1956): A description of 34 ova within the first 17 days of development. *Am. J. Anat.* 98, 435-491.
- Hewitt K, Beer AE and Grinnel F (1979): Disappearance of anionic sites from the surface of rat the rat endometrial epithelium at the time of implantation. *Biol. Reprod.* 21, 691-707.
- Hey NA and Aplin JD (1996): Sialyl Lewis x and sialyl Lewis a are expressed by human endometrial MUC1. *Glyconjug. J.* 13, 769-779.
- Hey NA, Graham RA, Seif MW and Aplin JD (1994): The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. *J. Clin. Endocrinol. Metab.* 78, 337-342.
- Hey NA, Li TC, Devine PL and Aplin JD (1995): MUC1 in secretory phase endometrium: expression in precisely date biopsies and flushings from normal and recurrent miscarriage patients. *Hum. Reprod.* 10, 2655-2662.
- Heymann D, Hard J, Ringear S, Blanchard F, Lassort D, Raher S and Godard A (1996): Modulation of integrin α -v- β -1 expression on human tumor cells by leukaemia inhibitory factor (LIF) and oncostatin M (OSM). *Bull. Cancer.* 83, 13-21.
- Hierck BP, Thorsteinsdottir S, Niessen CM, Freund E, Iperen LV, Feyen A, Hogervorst F, Poelmann RE, Mummery CL and Sonnenberg A (1993): Variants of the α 6 β 1 laminin receptor in early murine development. *Cell. Adhes. Comm.* 1, 33-53.
- Higashiyama S, Abraham JA and Klagsbrun M (1993): Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence of interactions with cell surface heparan sulphate. *J. Cell Biol.* 122, 933-940.
- Hoadley ME, Seif MW and Aplin JD (1990): Menstrual-cycle-dependent expression of keratan sulphate in human endometrium. *Biochem. J.* 266, 757-763.
- Hochfeld A, Beier HM and Denker HW (1990): In: Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. Trophoblast research 4. (ed. Denker HW & Aplin JD). Plenum Press, New York.

- Hofmann GE, Drews MR, Scott RT, Navot D, Heller D and Deligdisch L (1992): Epidermal growth factor and its receptors in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. *J. Clin. Endocrinol. Metab.* 74, 981-988.
- Honda S, Tmoyama Y, Pelletier AJ, Annis D, Honda Y, Orchekowski R, Ruggeri Z and Kunick TJ (1995): Topography of ligand induced binding sites, including a novel cation-sensitive epitope (AP5) at the amino terminus, of the human integrin $\beta 3$ subunit. *J. Biol. Chem.* 270, 11947-11954.
- Hou Q, Paria BC, Mui C, Dey SK and Gorski J (1996): Immunolocalisation of estrogen receptor protein in the mouse blastocyst during normal and delayed implantation. *Proc. Natl. Acad. Sci. USA.* 93, 2376-2381.
- Hsu Y-C (1971): Post-blastocyst differentiation in vitro. *Nature.* 231, 100-102.
- Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ and Ginsberg MH (1996): Breaking the integrin hinge. *J. Biol. Chem.* 271, 6571-6574.
- Humphries MJ (1996): Integrin activation: the link between ligand binding and signal transduction. *Curr. Op. Cell Biol.* 8, 632-640.
- Humphries MJ (1990): The molecular basis and specificity of integrin-ligand interactions. *J. Cell Sci.* 97, 585-592.
- Hunt JS, Chen HL, Hu XL and Tabibzadeh S (1992): Tumour necrosis factor-alpha messenger ribonucleic acid and protein in human endometrium. *Biol. Reprod.* 47, 141-147.
- Huovila APJ, Almeida EAC and White JM (1996): ADAMs and cell fusion. *Curr. Biol.* 8, 692-699.
- Hyafil F, Morello D, Babinet C and Jacobs F (1980): A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell.* 21, 927-934.
- Hynes RO (1996): Targeted mutations in cell adhesion genes: what have we learned from them? *Devel. Biol.* 180, 402-412.
- Hynes RO (1987): Integrins: a family of cell surface receptors. *Cell.* 48, 549-554.
- Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Wasburn TF, McLachlan JA and Korach KS (1992): Coupling of dual signalling pathways: epidermal growth factor action involves the estrogen receptor. *Proc. Natl. Acad. Sci. USA* 89, 4658-4662.
- Illera MJ, Das SK, Dey SK, Yuan L, Stewart C, Cullinan E and Lessey BA (1997): The $\alpha v \beta 3$ vitronectin receptor in the mouse uterus: a potential role during implantation. *J. Soc. Gynecol. Invest.* 4, A216.
- Iruela-Arispe ML, Porter P, Bornstein P and Sage EH (1996): Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium. *J. Clin. Invest.* 97, 403-412.

- Iwahashi M, Muragaki Y, Ooshima A, Yamoto M and Nakano R (1996): Alteration in distribution and composition of the extracellular matrix during decidualization of the human endometrium. *J. Repro. Fertil.* 108, 147-155.
- Izzard CS and Lochner LR (1976): Cell-to-substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique. *J. Cell Sci.* 21, 129-159.
- Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D, Holborow EJ (1982): Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immun. Methods.* 55, 231-242.
- Johnson MH, McConnell J and Van Blerkom J (1984): Programmed development in the mouse embryo. *J. Embryol. Exp. Morphol. Supp.* 83, 197-231.
- Jones JI, Prevette T, Gockerman A and Clemmons DR (1996): Ligand occupancy of the $\alpha v \beta 3$ integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA.* 93, 2482-2487.
- Julian J, Chiquet-Ehrismann R, Erickson HP and Carson DD (1994): Tenascin is induced at implantation sites in the mouse uterus and interferes with epithelial cell adhesion. *Devel.* 120, 661-671.
- Juliano D, Wang Y, Marcinkiewicz C and Rosenthal LA (1996): Disintegrin interaction with alpha v beta 3 integrin on human umbilical vein endothelial cells: expression of ligand-induced binding site on beta 3 subunit. *Exp. Cell Res.* 225, 132-142.
- Kadmon G and Altevogt P (1997): The cell adhesion molecule L1: species- and cell-type-dependent multiple binding mechanisms. *Differen.* 61, 143-150.
- Kaelin WG, Pallas DC, De Caprio JA, Kaye FJ and Livingston Dm (1991): Identification of cellular proteins that can interact with the T/E1A-binding region of the retinoblastoma gene product. *Cell.* 64, 521-532.
- Kapur S, Tamada H, Dey SK and Andrews GK (1992): Expression of insulin-like growth factor -I (IGF-I) and its receptor in the peri-implantation mouse uterus, and cell specific regulation of IGF-I gene expression by estradiol and progesterone. *Biol. Reprod.* 46, 208-219.
- Kauma S, Matt D, Strom S, Eierman D and Turner T (1990): Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. *Am. J. Obstet. Gynecol.* 163, 1430-1437.
- Kieffer N, Melchior C, Guinet JM, Michels S, Gouon V and Bron N (1996): Serine 752 in the cytoplasmic domain of the beta 3 integrin subunit is not required for alpha v beta 3 postreceptor signaling events. *Cell Adhes. Commun.* 4, 25-39.
- Kimber SJ (1990): Glycoconjugates and cell surface interactions in pre- and peri-implantation mammalian embryonic development. *Int. Rev. Cytol.* 120, 53-167.

- Kimber SJ and Lindenberg S (1990): Hormonal control of a carbohydrate epitope involved in implantation in mice. *J. Repro. Fertil.* 89, 13-21.
- Kimber SJ and Sidhu SS (1997): Control of expression of the H-type-I histo-blood group antigen in the murine endometrial epithelium and its role in blastocyst adhesion. *Hum. Reprod.* 12, 58-64.
- Kimber SJ, Bentley J, Ciemerych M, Moller CJ and Bock E (1994a): Expression of N-CAM in fertilized pre- and periimplantation and parthenogenetically activated mouse embryos. *Eur. J. Cell Biol.* 63, 102-113.
- Kimber SJ, Brown DG, Pahlsson P and Nilsson B (1993): Carbohydrate antigen expression in murine embryonic stem cells and embryos. *Histochem. J.* 25, 628-641.
- Kimber SJ, Illingworth IM and Glasser SR (1995): Expression of carbohydrate antigens in the rat uterus during early pregnancy and after ovariectomy and steroidal replacement. *J. Repro. Fertil.* 103, 75-87.
- Kimber SJ, Lindenberg S and Lundblad A (1988): Distribution of some Gal β 1-3(4)GlcNAc related carbohydrate antigens on the mouse uterine epithelium in relation to the peri-implantation period. *J. Repro. Immun.* 12, 297-313.
- Kimber SJ, White S, Cook A and Illingworth I (1994b): In, *Gamete and embryo quality* (ed. Mastrianni Jr. L). Parthenon Publishing Group, London.
- King LA and Posse RD (1992): In, *The baculovirus expression system: a laboratory guide* (ed. King & Posse). Chapman & Hall Ltd., London.
- Kitazawa S, Ross FP, McHugh K and Teitelbaum SL (1995): Interleukin-4 induces expression of the integrin α v β 3 via transactivation of the β 3 gene. *J. Biol. Chem.* 270, 4115-4119.
- Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L and Andreasen PA (1997): Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp. Cell Res.* 232, 420-429.
- Klentzeris LD, Bulmer JN, Trejdosiewicz LK, Morrison L and Cooke ID (1993): Beta-1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. *Hum. Reprod.* 8, 1223-1230.
- Knoth M and Larsen JF (1972): Ultrastructure of a human implantation site. *Acta. Obstet. Gynecol. Scand.* 51, 385-388.
- Knox P (1984): Kinetics of cell spreading in the presence of different concentrations of serum or fibronectin-depleted serum. *J. Cell Sci.* 71, 51-59.
- Kojima K, Kanzaki H, Iwai M, Hatayama H, Fujimoto M, Inoue T, Horie K, Nakayama H, Fujita J and Mori T (1994): Expression of leukemia inhibitory factor in human endometrium and placenta. *Biol. Reprod.* 50, 882-887.

- Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H and Kohler G (1994): Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368, 339-342.
- Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW and Smith EP (1996): Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog. Horm. Res.* 51, 159-186.
- Kornberg LJ, Earp HS, Turner CE, Prockop C and Juliano RL (1991): Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of $\beta 1$ integrins. *Proc. Natl. Acad. Sci. USA.* 88, 8392-8396.
- Kratzschmar J, Lum L and Blobel CP (1995): Metargidin, a membrane-anchored metalloprotease-disintegrin protein with an RGD integrin binding sequence. *J. Biol. Chem.* 271, 4593-4596.
- Kreitmann B, Bugat R and Bayard F (1979): Estrogen and progestin regulation of the progesterone receptor concentration in human endometrium. *J. Clin. Endocrinol. Metab.* 49, 926-929.
- Laemmli UK (1970): Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature.* 227, 680.
- Lafrenie RM and Yamada KM (1996): Integrin-dependent signal transduction. *J. Cell. Biochem.* 61, 543-553.
- Lam SCT (1992): Isolation and characterisation of a chymotryptic fragment of platelet glycoprotein IIb-IIIa retaining Arg-Gly-Asp binding activity. *J. Biol. Chem.* 267, 5649-5655.
- Larue L, Ohsugi M, Hirschenhain J and Kemler R (1994): E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc. Natl. Acad. Sci. USA.* 91, 8263-8267.
- Lavranos TC, Rathjen PD and Seamark RF (1995): Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. *J. Reprod. Fertil.* 105, 331-8.
- Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H and Hynes RO (1998): Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest.* 101, 982-992.
- Lawler J, Weinstein R and Hynes RO (1988): Cell attachment to thrombospondin: The role of Arg-Gly-Asp, calcium, and integrin receptors. *J. Cell Biol.* 107, 2351-2361.
- Leavitt WW, Cobb AD and Takeda A (1987): Progesterone-modulation of estrogen action: rapid down regulation of nuclear acceptor sites for the estrogen receptor. *Adv. Exp. Med. Biol.* 230, 49-78.
- Lee J-O, Rieu P, Arnaout MA and Liddington R (1995): Crystal structure of the A-domain from the α -subunit of $\beta 2$ integrin complement receptor type 3 (CR3, CD11b/CD18). *Cell.* 80, 631-638.

- Lee YJ and Gorski J (1996): Estrogen-induced transcription of the progesterone receptor gene does not parallel estrogen receptor occupancy. *Proc. Natl. Acad. Sci. USA.* 93, 15180-15184.
- Lehmann M, Rigot V, Seidah NG, Marvaldi J and Lissitzky (1996): Lack of integrin alpha-chain endoproteolytic cleavage in furin-deficient human colon adenocarcinoma cells LoVo. *Biochem. J.* 317, 803-809.
- Lessey BA, Albeda S, Buck CA, Castelbaum AJ, Yeh I, Kohler M and Berchuck A (1995a): Distribution of integrin cell adhesion molecules in endometrial cancer. *Am. J. Pathol.* 146, 717-726.
- Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW and Sun J (1994a): Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil. Steril.* 62, 497-506.
- Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W and Strom BL (1994b): Aberrant integrin expression in the endometrium of women with endometriosis. *J. Clin. Endocrinol. Metab.* 79, 643-649.
- Lessey BA, Castelbaum AJ, Sawin SJ and Sun J (1995b): Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil. Steril.* 63, 535-542.
- Lessey BA, Damjanovich L, Coutifaris C, Castelbaum A, Albeda SM and Buck CA (1992): Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J. Clin. Invest.* 90, 188-195.
- Lessey BA, Ilesanmi AO, Lessey MA, Riben M, Harris JE and Chwalisz K (1996): Luminal and glandular endometrial epithelium express integrins differentially throughout the menstrual cycle: implications for implantation, contraception, and infertility. *Am. J. Reprod. Immunol.* 35, 195-204.
- Levenberg S, Katz B-Z, Yamada KM and Geiger B (1998): Long-range and selective autoregulation of cell-cell or cell-matrix adhesion by cadherin or integrin ligands. *J. Cell Sci.* 111, 347-357.
- Li TC, Warren MA, Hill CJ and Saravelos H (1994): In, *The human endometrium* (ed. Bulletti C, Guroide E and Flamigni C). The New York Academy of Sciences, New York.
- Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM and Hogan BL (1998): Altered wound healing in mice lacking functional osteopontin gene (*spp1*). *J Clin Invest.* 101, 1468-1478.
- Liaw L, Skinner MP, Raines EW, Ross R, Cheresch DA, Schwartz SM and Giachelli CM (1995): The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. *J. Clin. Invest.* 95, 713-724.
- Lin ECK, Ratnikov BI, Tsai PM, Gonzalez ER, McDonald S, Pelletier AJ and Smith JW (1997): Evidence that the integrin $\beta 3$ and $\beta 5$ subunits contain a metal ion-dependent adhesion site-like motif but lack an I domain. *J. Biol. Chem.* 272, 14236-14243.

- Lindberg FP, Gresham HD, Reinhold MI and Brown EJ (1996): Integrin-associated protein immunoglobulin domain is necessary for efficient vitronectin bead binding. *J. Cell Biol.* 134, 1313-1322.
- Lindenberg S (1991): Experimental studies on the initial trophoblast endometrial interaction. *Dan. Med. Bull.* 38, 371-380.
- Lindenberg S, Hyttel P, Sjogren A and Greve T (1989): A comparative study of attachment of human, bovine and mouse blastocysts to uterine epithelial monolayers. *Hum. Reprod.* 4, 446-456.
- Lindenberg S, Kimber SJ, Kallin E (1990): Carbohydrate binding properties of mouse embryos. *J. Repro. Fertil.* 89, 431-439.
- Lindenberg S, Pedersen B, Hamberger L and Kimber SJ (1992): Models for human implantation derived from implantation in vitro. *Reprod. Fertil. Dev.* 4, 653-670.
- Lindenberg S, Sundberg K, Kimber SJ and Lundblad A (1988): The milk oligosaccharide, lacto-N-fucopentaose I, inhibits attachment of mouse blastocysts on endometrial monolayers. *J. Repro. Fertil.* 83, 149-158.
- Liu KY, Timmons S, Lin YZ and Hawiger J (1996a): Identification of a functionally important sequence in the cytoplasmic tail of integrin beta 3 by using cell-permeable peptide analogs. *Proc Natl. Acad. Sci. USA.* 93, 11819-11824.
- Liu S, Hoke D, Julian J and Carson DD (1997): Heparin/heparan sulfate (HP/HS) interacting protein (HIP) supports cell attachment and selective, high affinity binding of HP/HS. *J. Biol. Chem.* 272, 25856-25862.
- Liu S, Smith SE, Julian J, Rohde LH, Karin NJ and Carson DD (1996b): cDNA cloning and expression of HIP, a novel cell surface heparan sulfate/heparin-binding protein of human uterine epithelial cells and cell lines. *J. Biol. Chem.* 271, 11817-11823.
- Loftus JC, Halloran CE, Ginsberg MH, Feigen LP, Zablocki JA and Smith JW (1996): The amino-terminal one-third of alpha IIb defines the ligand recognition specificity of integrin alpha IIb beta 3. *J. Biol. Chem.* 271, 2033-2039.
- Loftus JC, Smith JW and Ginsberg MH (1994): Integrin-mediated cell adhesion: the extracellular face. *J. Biol. Chem.* 269, 25235-25238.
- Loke YW, Gardner L, Burland K and King A (1989): Laminin in human trophoblast-decidua interaction. *Hum. Reprod.* 4, 457-463.
- Lopata A, Kohlman DJ, Bowes LG and Watkins WB (1995): Culture of marmoset blastocysts on Matrigel: A model of differentiation during the implantation period. *Anat. Rec.* 241, 469-486.
- Lowry OH, Rosenbrough NJ, Farr AI and Randall RS (1951): Protein measurement with the folin Phenol reagent. *J. Biol. Chem.* 193, 265-268.

- Lovely LP, Odom LD, Fritz Ma, Meyer WR, Harris JE and Lessey BA (1997): Correlation of $\alpha\text{v}\beta 3$, serum progesterone and endometrial thickness with histologic dating. *J. Soc. Gynecol. Invest.* 4, A384.
- Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS and Smithies O (1993): Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. USA.* 90, 11162-11166.
- Luetteke NC, Qiu T-H, Peiffer RL, Oliver P, Smithies O and Lee DC (1993): TGF- α deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* 73, 263-278.
- Lui J-P, Baker J, Perkins AS, Robertson EA and Efstratiadis A (1993): Mice carrying null mutations of the genes encoding insulin-like growth factor I (IGF-I) and type I IGF receptor. *Cell* 75, 59-72.
- Lydon JP, DeMayo FJ, Conneely OM and O'Malley BW (1996): Reproductive phenotypes of the progesterone receptor null mutant mouse. *J. Steroid. Biochem. Mol. Biol.* 56, 1-6.
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM and O'Malley BW (1995): Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & Devel.* 9, 2266-2278.
- Mackay CR (1994): Expression and modulation of CD44 variant isoforms in humans. *J. Cell Biol.* 124, 71-82.
- Malak TM and Bell SC (1994): Differential expression of the integrin subunits in human fetal membranes. *J. Reprod. Fertil.* 102, 269-276.
- Marchetti D, Liu S, Spohn WC and Carson DD (1997): Heparanase and a synthetic peptide of heparan sulfate-interacting protein recognize common sites on cell surface and extracellular matrix heparan sulfate. *J. Biol. Chem.* 272, 15891-15897.
- Marguerie GA, Plow EF and Edgington TS (1979): Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 254, 5357-5363.
- Martin L and Finn CA (1968): Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus. *J. Endocrinol.* 41, 363-371.
- Maruo T, Matsuo H, Otani T and Mochizuki M (1997): In, *Trophoblast research 9* (Hata T, Takayama M and Taki I). University of Rochester press, NY.
- Massague J and Pandiella A (1993): Membrane anchored growth factors. *Ann. Rev. Biochem.* 62, 515-541.
- Massai MR, Bergeron C, Martel D, de Ziegler D, Meduri G, Pstchoyos A, Frydman R and Bouchard P (1993): Physiological oestradiol and progesterone replacement cycles in women with ovarian failure: a model to study endometrial maturation and sex steroid receptor regulation by exogenous hormones. *Hum. Reprod.* 8, 1828-1834.

- McCormack JT and Greenwald GS (1974): Progesterone and oestradiol-17 β concentrations in the peripheral plasma during pregnancy in the mouse. *J. Endocrinol.* 62, 101-107.
- McDonnell DP and Goldman ME (1994): RU486 exerts antiestrogenic activities through a novel progesterone receptor A form-mediated mechanism. *J. Biol. Chem.* 269, 11945-11949.
- McEver RP, Baenziger JU and Majerus PW (1982): Isolation and structural characterization of the polypeptide subunits of membrane glycoprotein IIb-IIIa from human platelets. *Blood.* 59, 80-85.
- McLean JW, Vestal DJ, Cheresch DA and Bodary SC (1990): cDNA sequence of the human $\beta 5$ subunit. *J. Biol. Chem.* 265, 17126-17131.
- Mehta RJ, Beate D, Brown A, Cullen E, Jonczyk A, Gussow D, Luckenbach GA and Goodman SL (1998): Transmembrane-truncated $\alpha v \beta 3$ integrin retains high affinity for ligand binding: evidence for an 'inside-out' suppressor? *Biochem. J.* 330, 861-869.
- Memmo LM and McKeown-Longo P (1998): The $\alpha v \beta 5$ integrin functions as an endocytic receptor for vitronectin. *J. Cell Sci.* 11, 425-433.
- Metzner B, Barbisch M, Bachmann F, Czech W and Norgauer J (1996): Evidence of the involvement of phosphatidylinositol 3-kinase in the migration, actin stress fiber formation, and $\alpha v \beta 3$ -integrin-mediated adherence of human melanoma cells. *J. Invest. Dermatol.* 107, 597-602.
- Milner R and French-Constant C (1994): A developmental analysis of oligodendroglial integrins in primary cells: changes in αv -associated β subunits during differentiation. *Devel.* 120, 3497-3506.
- Miyagoe Y, Hanaoka K, Nonaka I, Hayasaka M, Nabeshima Y, Arahata K, Nabeshima Y and Takeda S (1997): Laminin $\alpha 2$ chain-null mutant mice by targeted disruption of the *Lama2* gene: a new model of merosin (laminin-2)-deficient congenital muscular dystrophy. *FEBS Lett.* 415, 33-39.
- Miyamoto S, Akiyama SK and Yamada KM (1995): Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science.* 267, 883-885.
- Montgomery AM, Becker JC, Siu CH, Lemmon VP, Cheresch DA, Pancook JD, Zhao X and Reisfeld RA (1996): Human neural cell adhesion molecule L1 and rat homologue NILE are ligands for integrin $\alpha v \beta 3$. *J. Cell Biol.* 132, 475-485.
- Mooney DJ, Langer R and Ingber DE (1995): Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix. *J. Cell Sci.* 108, 2311-2320.
- Morris JE and Potter SW (1990): In: Trophoblast invasion and endometrial receptivity: novel aspects of the cell biology of embryo implantation. *Trophoblast research* 4. (ed. Denker HW & Aplin JD). Plenum Press, New York.

- Mossman HW (1987): Vertebrate fetal membranes. Rutgers University Press, New Brunswick.
- Mould AP (1996): Getting integrins into shape: recent insights into how integrin activity is regulated by conformational changes. *J. Cell Sci.* 109, 2613
- Mould AP, Akiyama SK and Humphries MJ (1995a): Regulation of integrin alpha 5 beta 1-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn^{2+} , Mg^{2+} , and Ca^{2+} . *J. Biol. Chem.* 270, 26270-26277.
- Mould AP, Askari JA, Aota S, Yamada KM, Irie A, Takada Y, Mardon HJ and Humphries MJ (1997): Defining the topology of integrin alpha5beta1-fibronectin interactions using inhibitory antibodies. Evidence that the synergy sequence of fibronectin is recognised by the amino-terminal repeats of the alpha5 subunit. *J. Biol. Chem.* 272, 17283-17292.
- Mould AP, Garrat AN, Askari JA, Akiyama SK and Humphries MJ (1995b): Identification of a novel anti-integrin monoclonal antibody that recognises a ligand-induced binding site epitope on the beta 1 subunit. *FEBS Lett.* 363, 118-122.
- Mulholland J, Aplin JD, Ayad S, Hong L and Glasser SR (1992): Loss of collagen type VI from rat endometrial stroma during decidualization. *Biol. Reprod.* 46, 1136-1143.
- Murphy CR, Swift JG, Mukherjee TM and Rogers AW (1982): Changes in the fine structure of the apical plasma membrane of endometrial epithelial cells during implantation in the rat. *J. Cell Sci.* 55, 1-12.
- Nachtigall MJ, Klinman HJ, Feinberg RF, Olive DL, Engin O and Arici A (1996): The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *J. Clin. Endocrinol. Metab.* 81, 801-806.
- Nelson KG, Takahashi T, Bossert NL, Walmer DK and McLachlan JA (1991): Epiderma; growth factor replaces estrogen in the stimulation of femal genital-tract growth and differentiation. *Proc. Natl. Acad. Sci. USA* 88, 21-25.
- Newham P, Craig SE, Seddon GN, Schofield NR, Rees A, Edwards RM, Jones EY and Humphries MJ (1997): $\alpha 4$ integrin binding interfaces on VCAM-1 and MAdCAM-1. *J. Biol. Chem.* 272, 19429-19440.
- Nikkari L, Haapasalmi K, Aho H, Torvinen A, Sheppard D, Larjava H and Heino J (1995): Localisation of the αv subfamily of integrins and their putative ligands in synovial lining cell layer. *J. Rheumatol.* 22, 16-23.
- Nilsson O (1966): Structural differentiation of luminal membrane in rat uterus during normal and experimental implantation. *Z. Anat. Entwickl.* 125, 152-159.
- Nishimura SL, Sheppard D and Pytela R (1994): Integrin $\alpha v \beta 8$. *J. Biol. Chem.* 269, 28708-28715.
- Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR and Merlie JP (1995): The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. *Nat Genet.* 10, 400-406.

- Nolan LA and Morgan AJ (1995): The Epstein-Barr virus open reading frame BDLF3 codes for a 100-150kDa glycoprotein. *J. Gen. Virol.* 76, 1381-1392.
- Noyes RW, Hertig AT and Rock J (1950): Dating the endometrial biopsy. *Fertil. Steril.* 1, 3-25.
- Omigbodun A, Daiter E, Walinsky D, Fisher L, Young M, Hiyer J and Coutifaris C (1995): In, Osteopontin: role in cell signalling and adhesion (ed. Denhardt D, Butler TW, Chambers AF and Senger DR). The New York Academy of Sciences, New York.
- Omigbodun A, Ziolkiewicz P, Tessler C, Hoyer JR and Coutifaris C (1997): Progesterone regulates osteopontin expression in human trophoblasts: a model of paracrine control in the placenta? *Endocrinol.* 138, 4308-4315.
- O'Shea KS, Liu LHJ, Kinnunen LH and Dixit VM (1990): Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo. *J. Biol. Chem.* 111, 2713-2723.
- O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ and Ginsberg MH (1994): Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* 124, 1047-1059.
- Panetti TS and McKeown-Longo PJ (1993): The $\alpha v\beta 5$ integrin receptor regulates receptor-mediated endocytosis of vitronectin. *J. Biol. Chem.* 268, 11492-11495.
- Panetti TS, Wilcox SA, Horzempa C and McKeown-Longo PJ (1995): $\alpha v\beta 5$ integrin receptor-mediated endocytosis of vitronectin is protein kinase C-dependent. *J. Biol. Chem.* 270, 18593-18597.
- Paria BC and Dey SK (1990): Preimplantation embryos in vitro: cooperative interactions among embryos and role of growth factors. *Proc. Natl. Acad. Sci. USA* 87, 4756-4760.
- Paria BC, Das SK, Andrews GK and Dey SK (1993a): Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. *Proc. Natl. Acad. Sci. USA* 90, 55-59.
- Paria BC, Huet-Hudson YM and Dey SK (1993b): Blastocyst's state of activity determines the window of implantation in the receptive mouse uterus. *Devel. Biol.* 99, 10159-10162.
- Paria BC, Jones KL, Flanders KC and Dey SK (1992): Localization and binding of transforming growth factor-beta isoforms in mouse preimplantation embryos and in delayed and activated blastocysts. *Dev. Biol.* 151, 91-104.
- Pasqualini R and Hemler ME (1994): Contrasting roles for the integrin $\beta 1$ and $\beta 5$ cytoplasmic domains in subcellular localization, cell proliferation, and cell migration. *J. Cell Biol.* 125, 447-460.
- Pasqualini R, Bodorova J, Ye S and Hemler ME (1993): Study of the structure, function and distribution of $\beta 5$ integrins using novel anti- $\beta 5$ monoclonal antibodies. *J. Cell Sci.* 105, 101-111.

- Pelletier Aj, Kunicki T, Quaranta V (1996): Activation of the integrin $\alpha v \beta 3$ involves a discrete cation-binding site that regulates conformation. *J. Biol. Chem.* 271, 1364-1370.
- Perrot-Appianat M, Groyer-Picard MT, Garcia E, Lorenzo F and Milgrom E (1988): Immunocytochemical demonstration of estrogen and progesterone receptors in muscle cells of uterine arteries in rabbits and humans. *Endocrinol.* 123, 1511-1519.
- Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M and Mak TW (1993): Mice deficient for the 55 kd tumour necrosis factor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73, 457-467.
- Piali L, Hammel P, Uherek C, Bachmann F, Gisler RH, Dunon D and Imhof BA (1995): CD31/PECAM-1 is a ligand for $\alpha v \beta 3$ integrin involved in adhesion of leukocytes to endothelium. *J. Cell Biol.* 130, 451-460.
- Pierschbacher MD and Ruoslahti E (1987): Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J. Biol. Chem.* 262, 17294-17298.
- Pijuan-Thompson V and Gladson C (1997): Ligation of integrin $\alpha 5 \beta 1$ is required for internalization of vitronectin by integrin $\alpha v \beta 3$. *J. Biol. Chem.* 272, 2736-2743.
- Pimental RA, Julian J, Gendler SJ and Carson DD (1996): Synthesis and intracellular trafficking of Muc-1 and mucins by polarized mouse uterine epithelial cells. *J. Biol. Chem.* 271, 28128-28137.
- Plopper GE, McNamee HP, Dike LE, Bojanowski K and Ingber DE (1995): Convergence of integrin and growth factor receptor signalling pathways within the focal adhesion complex. *Molec. Biol. Cell.* 6, 1349-1365.
- Poirier F and Kimber SJ (1997): Cell surface carbohydrates and lectins in early development. *Mol. Hum. Reprod.* 3, 907-918.
- Pollard SW, Hunt JJ, Wiktar-Jedrzejczak W (1991): A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. *Devel. Biol.* 148, 273-283.
- Press MF, Udove JA and Greene GL (1988): Progesterone receptor distribution in the human endometrium. Analysis using monoclonal antibodies to the human progesterone receptor. *Am. J. Pathol.* 131, 112-124.
- Psychoyos A (1986): Uterine receptivity for nidation. *Ann. NY Acad. Sci.* 476, 36-42.
- Psychoyos A (1976): Hormonal control of receptivity for nidation. *J. Repro. Fertil. Supplement* 25, 17-28.
- Pullan S, Wilson J, Metcalfe A, Edwards G, Goberdhan N, Tilly J, Hickman J, Dive C and Streuli C (1996): Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. *J. Cell Sci.* 109, 631-642.

- Quarmby VE and Korach KS (1984): Differential regulation of protein synthesis by estradiol in uterine component tissues. *Endocrinol.* 115, 687-697.
- Quinn P, Barros C and Whittingham DG (1982): Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.* 66, 161-168.
- Raab G and Klagsbrun M (1997): Heparin-binding EGF-like growth factor. *Biochim. Biophys. Acta.* 1333, F179-F199.
- Raab G, Kover K, Paria BC, Dey SK, Ezzell RM and Klagsbrun M (1996): Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. *Devel.* 122, 637-645.
- Rai V, Hopkisson J, Kennedy S, Bergqvist A, Barlow DH and Mardon HJ (1996): Integrins $\alpha 3$ and $\alpha 6$ are differentially expressed in endometrium and endometriosis. *J. Pathol.* 180, 181-187.
- Ramsey EM, Houston ML and Harris JWS (1976): Interaction of the trophoblast and maternal tissue in three closely related primate species. *Am. J. Obstet. Gynecol.* 124, 647-652.
- Richardson A and Parsons T (1996): A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125FAK. *Nature.* 380, 538-540.
- Rider V and Psychoyos A (1994): Inhibition of progesterone receptor function results in loss of basic fibroblast growth factor expression and stromal cell proliferation during uterine remodelling in the pregnant rat. *J. Endocrinol.* 140, 239-249.
- Rider V, Carlone DL, Witrock D, Cai C and Oliver N (1992): Uterine fibronectin mRNA content and localization are modulated during implantation. *Devel. Dynam.* 195, 1-14.
- Roberts DK, Walker NJ and Lavia LA (1988): Ultrastructural evidence of stromal/epithelial interactions in the human endometrial cycle. *Am. J. Obstet. Gynecol.* 158, 854-861.
- Rohde LH and Carson DD (1993): Heparin-like glycosaminoglycans participate in binding of a human trophoblastic cell line (JAR) to a human uterine epithelial cell line (RL95). *J. Cell. Physiol.* 155, 185-196.
- Rohde LH, Julian J, Babikania A and Carson DD (1996): Cell surface expression of HIP, a novel heparin/heparan sulfate binding protein of human yterine epithelial cells and cell lines. *J. Biol. Chem.* 271, 11824-11830.
- Romagnano L and Babiarz B (1993): Mechanisms of murine trophoblast interaction with laminin. *Biol. Reprod.* 49, 374-380.
- Rosa J-P and McEver RP (1989): Processing and assembly of the integrin, glycoprotein IIb-IIIa, in HEL cells. *J. Biol. Chem.* 264, 12596-12603.
- Rosenfeld MG and Joshi MS (1977): A possible role of a specific uterine fluid peptidase in implantation in the rat. *J. Reprod. Fertil.* 51, 137-139.

- Ruck P, Marzusch K, Kaiserling E, Horny HP, Dietl J, Geiselhart A, Handgretinger R and Redman CWG (1994): Distribution of cell adhesion molecules in decidua of early human pregnancy. *Lab. Invest.* 71, 94-101.
- Ruoslahti E (1997): Integrin as signaling molecules and targets for tumour therapy. *Kidney Int.* 51, 1413-1417.
- Ruoslahti E (1988): Fibronectin and its receptors. *Ann. Rev. Biochem.* 57, 375-413.
- Ruoslahti E and Pierschbacher MD (1987): New perspectives in cell adhesion: RGD and integrins. *Science.* 238, 491-497.
- Ruoslahti E, Noble NA, Kagami S and Border WA (1994): Integrins. *Kidney Int.* 44, S17-S22.
- Schaller MD and Parsons JT (1994): Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* 6, 705-710.
- Schatz F, Gordon RE, Laufer N and Gurpide E (1990): Culture of human endometrial cells under polarizing conditions. *Differen.* 42, 184-190.
- Schindler J and Sherman MI (1981): Effects of α -mannan on programming of mouse blastocyst development. *Devel. Biol.* 84, 332-340.
- Schlafke S and Enders AC (1975): Cellular basis of interaction between trophoblast and uterus at implantation. *Biol. Reprod.* 12, 41-65.
- Schlafke S, Welsh AO and Enders AC (1985): Penetration of the basal lamina of the uterine luminal epithelium during implantation in the rat. *Anat. Rec.* 212, 47-56.
- Schultz JF and Armant DR (1995): β 1- and β 3- Class integrins mediate fibronectin binding activity at the surface of developing mouse peri-implantation blastocysts. *J. Biol. Chem.* 270, 11522-11531.
- Schwartz MA (1997): Integrins, oncogenes, and anchorage independence. *J. Cell Biol.* 139, 575-578.
- Seiffert D, Crain K, Wagner NV and Loskutoff DJ (1994): Vitronectin gene expression in vivo. *J. Biol. Chem.* 269, 19836-19842.
- Shen MM and Leder P (1992): Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. *Proc. Natl. Acad. Sci. USA* 89, 8240-8244.
- Sherman MI (1975): The culture of cells derived from mouse blastocysts. *Cell.* 5, 343-349.
- Sherman MI and Salomon DS (1975): In, *The developmental biology of reproduction* (ed. Markert CL & Papaconstantinou J). Academic Press, New York.

- Shiokawa S, Yoshimura Y, Nagamatsu S, Sawa H, Hanashi H, Koyama N, Katsumata Y, Nagai A and Nakamura Y (1996a): Function of $\beta 1$ integrins on human decidual cells during implantation. *Biol. Reprod.* 54, 745-752.
- Shiokawa S, Yoshimura Y, Nagamatsu S, Sawa H, Hanashi H, Oda T, Katsumata Y, Koyama N, and Nakamura Y (1996b): Expression of $\beta 1$ integrins in human endometrial and decidual cells. *J. Clin. Endocrinol. Metab.* 81, 1533-1540.
- Shiotani M, Noda Y and Mori T (1993): Embryo-dependent induction of uterine receptivity assessed by an in vitro model of implantation in mice. *Biol. Reprod.* 49, 794-801.
- Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G and Calvin D (1992): Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359, 693-699.
- Sibilia M and Wagner EF (1995): Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269, 234-238.
- Sillem M, Prifti S, Schmidt M, Rabe T and Runnebaum B (1997): Endometrial integrin expression is independent of estrogen or progestin treatment in vitro. *Fertil. Steril.* 67, 877-882.
- Simon C (1996): Potential molecular mechanisms for the contraceptive control of implantation. *Molec. Hum. Reprod.* 2, 475-480.
- Simon C, Frances A, Piquette GN, Danasouri IE, Zurawski G, Dang W and Polan ML (1994): Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinol.* 134, 521-528.
- Simon C, Gimeno MJ, Mercader A, Frances A, Velasco JG, Remohi J, Polan ML and Pellicer A (1996): Cytokines-adhesion molecules-invasive proteases. The missing paracrine/autocrine link in embryonic implantation. *Molec. Hum. Reprod.* 2, 405-424.
- Simon C, Gimeno MJ, Mercader A, O'Conner E, Polan ML and Pellicer A (1997a): Embryonic regulation of $\beta 3$ -integrin in human endometrial epithelial cells is mediated by IL-1. *J. Soc. Gynecol. Invest.* 4, A101.
- Simon KO, Nutt E, Abraham DG, Rodan GA and Duong LT (1997b): The $\alpha v \beta 3$ integrin regulates $\alpha 5 \beta 1$ -mediated cell migration toward fibronectin. *J. Biol. Chem.* 272, 29380-29389.
- Sjaastad MD and Nelson WJ (1997): Integrin-mediated calcium signaling and regulation of cell adhesion by intracellular calcium. *BioEssays.* 19, 47-55.
- Sjaastad MD, Lewis RS and Nelson WJ (1996): Mechanisms of integrin-mediated calcium signaling in MDCK cells: regulation of adhesion by IP3- and store-independent calcium influx. *Mol. Biol. Cell.* 7, 1025-1041.

- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB and Korach KS (1994): Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N. Engl. J. Med.* 331, 1056-1061.
- Smith JW and Cheresh DA (1988): The Arg-Gly-Asp binding domain of the vitronectin receptor. *J. Biol. Chem.* 263, 18726-18731.
- Smith JW, Vestal DJ, Irwin SV, Burke TA and Cheresh DA (1990): Purification and functional characterization of integrin α v β 5. An adhesion receptor for vitronectin. *J. Biol. Chem.* 265, 11008-11013.
- Smith SE, French MM, Julian J, Paria BC, Dey SK and Carson DD (1997): Expression of heparan sulfate proteoglycan (perlecan) in the mouse blastocyst is regulated during normal and delayed implantation. *Devel. Biol.* 184, 38-47.
- Snell GD and Stevens LC (1966): In, *Biology of the laboratory mouse* (ed. Green EL). McGraw-Hill, New York.
- Somkuti SG, Yuan L, Fritz MA and Lessey BA (1997): Epidermal growth factor and sex steroids dynamically regulate a marker of endometrial receptivity in Ishikawa cells. *J. Clin. Endocrin. Metab.* 82, 2192-2197.
- Spanswick C, Swann H, Behzad F, Vicovac LJ, Kimber SJ and Aplin JD (1995): Integrins of the α v family on the trophectodermal and endometrial cell surface. *Placenta.* 16, A68.
- Stephens LE, Sonne JE, Fitzgerald ML and Damsky CH (1993): Targeted deletion of β 1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* 123, 1607-1620.
- Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA and Damsky CH (1995): Deletion of β 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes & Devel.* 9, 1883-1895.
- Stewart CL (1994): In, *The human endometrium* (ed. Bulletti C, Guroide E and Flamigni C). The New York Academy of Sciences, New York.
- Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F and Abbondanzo SJ (1992): Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 359, 76-79.
- Stuiver I, Ruggeri Z and Smith JW (1996): Divalent cations regulate the organisation of integrins α v β 3 and α v β 5 on the cell surface. *J. Cell. Physiol.* 168, 521-531.
- Suehiro K, Smith JW and Plow EF (1996): The ligand binding recognition specificity of β 3 integrins. *J. Biol. Chem.* 271, 10365-10371.
- Sueoka K, Shiokawa S, Miyazaki T, Kuji N, Tanaka M and Yoshimura Y (1997): Integrins and reproductive physiology: expression and modulation in fertilization, embryogenesis, and implantation. *Fertil. Steril.* 67, 799-811.

- Sugimori T, Griffith DL and Arnaout MA (1997): Emerging paradigms of integrin ligand binding and activation. *Kidney Int.* 51, 1454-1462.
- Surveyor GA, Gendler SJ, Peberton L, Das SK, Chakraborty I, Julian J, Pimental RA, Wegner CC, Dey SK and Carson DD (1995): Expression and steroid hormone control of Muc-1 in the mouse uterus. *Endocrinol.* 136, 3639-3647.
- Sutherland AE, Calarco PG and Damsky CH (1993): Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Devel.* 119, 1175-1186.
- Sutherland AE, Calaro PG and Damsky CH (1988): Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth. *J. Cell Biol.* 106, 1331-1348.
- Symonds EM (1992): *Essential Obstetrics and Gynaecology*. Churchill Livingstone, NY.
- Tabibzadeh S (1992): Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. *Hum. Reprod.* 7, 876-882.
- Tabibzadeh S and Babaknia A (1995): The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Molec. Hum. Reprod.* 10, 1579-1602.
- Tabibzadeh S and Sun XZ (1992): Cytokine expression in human endometrium throughout the menstrual cycle. *Hum. Reprod.* 7, 1214-1221.
- Tachi S, Tachi C and Lindner HR (1970): Ultrastructural features of blastocyst attachment and trophoblastic invasion in the rat. *J. Reprod. Fertil.* 21, 37-56.
- Tahiliani PD, Singh L, Auer KL and LaFemme SE (1997): The role of conserved amino acid motifs within the integrin $\beta 3$ cytoplasmic domain in triggering focal adhesion kinase phosphorylation. *J. Biol. Chem.* 272, 7892-7898.
- Takeichi M (1988): The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Devel.* 102, 639-655.
- Tamada H, Das SK, Andrews GK and Dey SK (1991): Cell-type-specific expression of transforming growth factor- α in the mouse uterus during peri-implantation period. *Biol. Reprod.* 45, 365-372.
- Tamada H, McMaster MT, Flanders KC, Andrews GK and Dey SK (1990): Cell type specific expression of TGF- $\beta 1$ in the mouse uterus during peri-implantation period. *Molec. Endocrinol.* 4, 965-972.
- Tang B, Guller S and Gurpide E (1994): In, *The human endometrium* (ed. Bulletti C, Guroide E and Flamigni C). The New York Academy of Sciences, New York.
- Tarone G, Russo MA, Hirsch E, Odorisio T, Altruda F, Silengo L and Siracusa G (1993): Expression of $\beta 1$ integrin complexes on the surface of unfertilized mouse oocyte. *Devel.* 117, 1369-1375.

- Taskin O, Brown RW, Young DC, Poindexter AN and Wiehle RD (1994): High doses of oral contraceptives do not alter endometrial $\alpha 1$ and $\alpha V\beta 3$ integrins in the late implantation window. *Fertil. Steril.* 61, 850-855.
- Thie M, Fuchs P, Butz S, Sieckmann F, Hoschutzky H, Kemler R and Denker HW (1996): Adhesiveness of the apical surface of uterine epithelial cells: the role of junctional complex integrity. *Eur. J. Cell Biol.* 70, 221-232.
- Thie M, Harrach-Ruorecht B, Sauer H, Fuchs P, Albers A and Denker HW (1995): Cell adhesion to the apical pole of epithelium: a function of cell polarity. *Eur. J. Cell Biol.* 66, 180-191.
- Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourtou T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey RJ and Magnuson T (1995): Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269, 230-233.
- Timpl R and Brown JC (1994): The laminins. *Matrix Biol.* 14, 275-281.
- Todt JC, Yang Y, Lei J, Lauria MR, Sorokin Y, Cotton DB and Yelian FD (1996): Effects of tumour necrosis factor- α on human trophoblast cell adhesion and motility. *Am. J. Reprod. Immunol.* 36, 65-71.
- Tran TQ and Illsley NP (1997): Role of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in human endometrial-blastocyst attachment. *J. Soc. Gynecol. Invest.* 4, A432.
- Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F and Giguere V (1997): Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol. Endocrinol.* 11, 353-365.
- Tsark EC, Adamson ED, Withers GE and Wiley LM (1997): Expression and function of amphiregulin during murine preimplantation development. *Mol. Reprod. Devel.* 47, 271-283.
- Tsutsumi O and Oka T (1987): Epidermal growth factor deficiency during pregnancy causes abortion in mice. *Am. J. Obstet. Gynecol.* 156, 241-244.
- Tuckwell DS, Weston SA and Humphries MJ (1993): Integrins: a review of their structure and mechanisms of ligand binding. *Symp. Soc. Exp. Biol.* 47, 107-136.
- Tulsiani DRP, Yoshida-Komiya H and Araki Y (1997): Mammalian fertilization: a carbohydrate-mediated event. *Biol. Reprod.* 57, 487-494.
- Turpeenniemi-Hujanen T, Feinberg RF, Kauppila A and Puistola U (1995): Extracellular matrix interactions in early human embryos: implications for normal implantation events. *Fertil. Steril.* 64, 132-138.
- Turpeenniemi-Hujanen T, Ronnberg L, Kauppila A and Puistola U (1992): Laminin in the human embryo implantation: analogy to the invasion by malignant cells. *Fertil. Steril.* 58, 105-113.

- Valdizan MC, Julian J and Carson DD (1992): WGA-binding mucin glycoproteins protect the apical cell surface of mouse uterine epithelial cells. *J. Cell Physiol.* 151, 451-465.
- van der Linden PJQ, Erkens HWH, de Goeij AFPM, Evers JLH and Dunselman GAJ (1995): Expression of cadherins and integrins in human endometrium throughout the menstrual cycle. *Fertil. Steril.* 63, 1210-1216.
- van de Linden PJQ, Goeij AFPM, Dunselman GAJ, van der Linden EPM, Ramaekers FCS and Evers JLH (1994): Expression of integrins and E-cadherin in cells from menstrual effluent, endometrium, peritoneal fluid, peritoneum, and endometriosis. *Fertil. Steril.* 61, 85-90.
- Vicovac Lj, Jones CJP and Aplin JD (1995): Trophoblast differentiation during formation of anchoring villi in model of the early human placenta in vitro. *Placenta.* 16, 46-56.
- Wang A, Yokosaki Y, Ferrando R, Balmes J and Sheppard D (1996): Differential regulation of airway epithelial integrins by growth factors. *Am. J. Respir. Cell. Mol. Biol.* 15, 664-672.
- Watanabe T, Fukaya T, Yajima A and Sasano H (1997): In, *Trophoblast research 9* (Hata T, Takayama M and Taki I). University of Rochester press, NY.
- Watanabe T, Shintani A, Nakata M, Shing Y, Folkman J, Igarashi K and Sasada R (1994): Recombinant human betacellulin, molecular structure, biological activities and receptor interaction. *J. Biol. Chem.* 269, 9966-9973.
- Watson AJ (1992): The cell biology of blastocyst development. *Mol. Reprod. Dev.* 33, 492-504.
- Wayner EA, Orlando RA and Cheresch DA (1991): Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute to cell attachment to vitronectin but differentially on the cell surface. *J. Cell Biol.* 113, 919-929.
- Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV and Chapman HA (1996): Regulation of integrin function by the urokinase receptor. *Science.* 273, 1551-1555.
- Weinacker A, Chen A, Agrez M, Cone RI, Nishimura S, Wayner E, Pytela R and Sheppard D (1994): Role of the integrin $\alpha v\beta 6$ in cell attachment to fibronectin. *J. Biol. Chem.* 269, 6940-6948.
- Wen DX, Xu YF, Mais DE, Goldman ME and McDonnell DP (1994): The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. *Mol. Cell Biol.* 14, 8356-8364.
- Weskamp G and Blobel P (1994): A family of cellular proteins related to snake venom disintegrins. *Proc. Natl. Acad. Sci. USA.* 91, 2748-2751.
- Wesseling J, van der Valk SW, Vos HL, Sonnenberg A and Hilkens J (1995): Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. *J. Cell Biol.* 129, 255-265.

- Wewer UM, Faber M, Liotta LA and Albrechtsen R (1985): Immunochemical and ultrastructural assessment of the nature of the pericellular basement membrane of human decidual cells. *Lab. Invest.* 53, 624-633.
- White S and Kimber SJ (1994): Changes in $\alpha(1-2)$ fucosyltransferase activity in the murine endometrial epithelium during the estrous cycle, early pregnancy and after ovariectomy and hormone replacement. *Biol. Reprod.* 50, 73-81.
- Williams MJ, Hughes PE, O'Toole TE and Ginsberg MH (1994): The inner world of cell adhesion: integrin cytoplasmic domains. *Trends Cell Biol.* 4, 109-112.
- Wilson O, Jacobs AL, Stewart S and Carson DD (1990): Expression of externally disposed heparin/heparan sulfate binding sites by uterine epithelial cells. *J. Cell Physiol.* 143, 60-67.
- Wippler J, Kouns WC, Schlaeger E-J, Kuhn H, Hadvary P and Steiner B (1994): The integrin $\alpha IIb-\beta 3$, platelet glycoprotein IIb-IIIa, can form a functionally active heterodimer complex without the cysteine-rich repeats of the $\beta 3$ subunit. *J. Biol. Chem.* 269, 8754-8761.
- Wolfsberg TG and White JM (1996): ADAMs in fertilization and development. *Devel. Biol.* 180, 389-401.
- Woodard AS, Garcia-Cardena G, Leong M, Madri JA, Sessa WC and Languino LR (1998): The synergistic activity of $\alpha v\beta 3$ integrin and PDGF receptor increases cell migration. *J. Cell Sci.* 111, 469-478.
- Wordinger RJ, Smith KJ, Bell C, and Chang IC (1994): The immunolocalization of basic FGF in the mouse uterus during the initial stages of embryo implantation. *Growth Factors* 11, 175-186.
- Wright PS, Saudek V, Owen TJ, Harbeson SL and Bitoni AJ (1993): An echistatin C-terminal peptide activates GPIIb/IIIa binding to fibrinogen, fibronectin, vitronectin and collagen type I and type IV. *Biochem. J.* 293, 263-267.
- Wu C, Hughes PE, Ginsberg MH and McDonald JA (1996): Identification of a new biological function for the integrin $\alpha v\beta 3$: initiation of fibronectin assembly. *Cell Adhes. Commun.* 4, 149-158.
- Wu TC, Wan YJ, Chung AE and Damjanov I (1983): Immunohistochemical localization of entactin and laminin in early mouse embryos and fetuses. *Devel. Biol.* 100, 496-505.
- Xu H, Christmas P, Wu XR, Wewer UM and Engvall E (1994): Defective muscle basement membrane and lack of M-laminin in the dystrophic dy/dy mouse. *Proc Natl Acad Sci USA.* 91, 5572-5576.
- Yamada KM and Geiger B (1997): Molecular interactions in cell adhesion complexes. *Curr. Opin. Cell Biol.* 9, 76-85.
- Yamagata T and Yamazaki K (1991): Implanting mouse embryos stain with LNF-1 bearing fluorescent probe at their mural trophectoderm side. *Biochem. Biophys. Res. Commun.* 181, 1004-1009.

- Yanagimachi R (1988): In, The physiology of reproduction (ed. Knobil E and Neill J). Raven Press, New York.
- Yang JT and Hynes RO (1996): Fibronectin receptor functions in early embryonic cells deficient in $\alpha 5\beta 1$ integrin can be replaced by αv integrins.
- Yang Z-M, Le S-P, Chen D-B, Yasukawa K and Harper MJK (1995): Expression patterns of leukemia inhibitory factor receptor (LIFR) and the gp130 receptor component in rabbit uterus during early pregnancy. *J. Reprod. Fertil.* 103, 249-255.
- Yaron Y, Botchan A, Amit A, Reuben R, Peyser M, David MP and Lessing JB (1994): Endometrial receptivity in the light of modern assisted reproductive technologies. *Fertil. Steril.* 62, 225-232.
- Yasunori Y (1997): Integrins: expression, modulation, and signalling in fertilization, embryogenesis and implantation.
- Yelian FD, Edgeworth NA, Dong LJ, Chung AE and Armant DR (1993): Recombinant entactin promotes mouse primary trophoblast cell adhesion and migration through the Arg-Gly-Asp (RGD) recognition sequence. *J. Biol. Chem.* 121, 923-929.
- Yelian FD, Todt JC, Eliraz Y, Carenno C, Svinarich DM, Song J, Deppe G and Diamond MP (1997): Altered expression of $\beta 3$ integrin in human endometrial cancer cells. *J. Soc. Gynecol. Invest.* 4, A358.
- Yelian FD, Yang Y, Hirata JD, Schultz JF and Armant DR (1995): Molecular interactions between fibronectin and integrins during mouse blastocyst outgrowth. *Molec. Reprod. Devel.* 41, 435-448.
- Ylanne J, Cheresch DA and Virtanen I (1990): Localisation of beta 1, beta 3, alpha 5, alpha v and alpha IIb subunits of the integrin family in spreading human erythroleukemia cells. *Blood.* 76, 570-577.
- Yoo HJ, Barlow DH and Mardon HJ (1997): Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. *Dev. Genet.* 21, 102-108.
- Yoshimura Y (1997): Integrins: expression, modulation, and signalling in fertilisation, embryogenesis and implantation. *Keio J. Med.* 46, 16-24.
- Zhang X, Shu MA, Harvey MB and Schultz GA (1996): Regulation of urokinase plasminogen activator production in implanting mouse embryo: effect of embryo interaction with extracellular matrix. *Biol. Reprod.* 54, 1052-1058.
- Zheng DQ, Fornardo M, Bofetiado CJM, Tallini G, Bosari S and Languino LR (1997): Modulation of cell proliferation by the integrin cytoplasmic domain. *Kidney Int.* 51, 1434-1440.
- Zheng X, Saunders TL, Camper SA, Samuelson LC and Ginsburg D (1995): Vitronectin is not essential for normal mammalian development and fertility. *Proc Natl Acad Sci USA.* 92, 12426-12430.

Zhu ZM, Kojima N, Stroud MR, Hakomori SI and Fenderson BA (1995): Monoclonal antibody directed to Le^y oligosaccharide inhibits implantation in the mouse. *Biol. Reprod.* 52, 903-912.

APPENDICES

Appendix 1 Immunocytochemistry reagents*Phosphate buffered saline (PBS)*

140mM NaCl
2.7mM KCl
10.1mM Na₂HPO₄
1.8mM K₂HPO₄

Gelvatol

144ml 0.02M NaHPO₄
~56ml 0.02MKH₂PO₄ (to make to pH 7.2)

Take 160ml of the above solution and add the following:

1.31g NaCl
0.1g Sodium azide
2.4g DABCO
40g Vinyl alcohol
80ml Glycerol

C8F12 hybridoma medium (1L)

500ml DMEM High Glucose (Gibco BRL)
500ml RPMI 1640 (Gibco BRL)
1% (v/v) Non-essential amino acids (Gibco BRL)
1% (v/v) Nutridoma-SR (Boehringer Mannheim)
1% (v/v) Sodium pyruvate (Gibco BRL)
Antibiotics (Gibco BRL)

Appendix 2 Molecular biology solutions

Lauria Bertini (L B) medium (1L)

950ml Deionised H₂O
10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl

LB agar

Add 15g/L bacto-agar to LB medium (prepared as above). Autoclave solution then cool to 50°C before adding antibiotics. Use ~30ml per 90mm plate.

SOC medium (1L)

950ml deionised H₂O
20g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl
20mM Glucose

Isopropylthio-β-D-galactoside (IPTG)

10ml distilled H₂O
2g IPTG

Tris EDTA (TE) buffer

10mM Tris-HCl (pH 8)
1mM EDTA

Glycerol stocks

To 0.85ml of bacterial culture add 0.15ml of sterile glycerol, mix well. Store at -80°C. To initiate a culture, take a stab of glycerol stock and streak onto an LB agar plate (containing appropriate antibiotics). Grow up culture from a single colony picked from this plate.

Tris acetate EDTA (TAE) buffer

40mM Tris

21mM Glacial acetic acid

5mM EDTA

*6X loading buffer*To double distilled H₂O, add the following:

40% (w/v) Sucrose

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol FF

STE buffer

0.1M NaCl

10mM Tris-HCl (pH 8)

1mM EDTA (pH 8)

Appendix 3 Biochemistry solutions*Polyacrylamide gel electrophoresis 10X running buffer (pH 8.3)*

30g/l Tris base

144g/l Glycine

10g/l SDS

Add 30mls of 10X stock to 270mls water prior to use, do not adjust pH.

Appendix 4 Outgrowth culture raw data and control statistics

Appendix 4.1 Stage of outgrowth achieved with differing culture conditions

| Substrate | Supplement | Total | | Non-outgrowing | | | | Outgrowing | |
|-----------|-----------------------|--------|-------|----------------|-------|----------|-------|------------|-------|
| | | number | | Unattached | | Attached | | Day 3 | Day 4 |
| | | Day 3 | Day 4 | Day 3 | Day 4 | Day 3 | Day 4 | | |
| VN | none | 402 | 147 | 28 | 2 | 85 | 8 | 289 | 137 |
| VN | IG | 32 | 25 | 1 | 0 | 4 | 2 | 27 | 23 |
| VN | α v | 75 | 70 | 34 | 28 | 28 | 12 | 13 | 30 |
| VN | β 1 | 96 | 92 | 13 | 9 | 21 | 6 | 62 | 77 |
| VN | α v+ β 1 | 27 | 24 | 3 | 4 | 20 | 14 | 4 | 6 |
| FN | none | 230 | 154 | 30 | 7 | 66 | 5 | 134 | 142 |
| FN | IG | 45 | 41 | 1 | 0 | 3 | 2 | 41 | 39 |
| FN | α v | 104 | 78 | 38 | 10 | 38 | 56 | 28 | 12 |
| FN | β 1 | 62 | 79 | 8 | 5 | 23 | 12 | 31 | 62 |
| FN | α v+ β 1 | 43 | 41 | 7 | 0 | 31 | 40 | 5 | 1 |
| EHS | none | 232 | 165 | 52 | 6 | 123 | 52 | 57 | 107 |
| EHS | IG | 24 | 23 | 5 | 1 | 14 | 6 | 5 | 16 |
| EHS | α v | 41 | 41 | 7 | 5 | 26 | 12 | 8 | 24 |
| EHS | β 1 | 81 | 77 | 69 | 59 | 12 | 18 | 0 | 0 |
| EHS | α v+ β 1 | 48 | 48 | 42 | 36 | 6 | 12 | 0 | 0 |
| TSP | none | 65 | 64 | 61 | 60 | 4 | 4 | 0 | 0 |
| TSP | α v | 21 | 21 | 21 | 21 | 0 | 0 | 0 | 0 |
| TSP | β 1 | 16 | 16 | 16 | 16 | 0 | 0 | 0 | 0 |
| TSP | α v+ β 1 | 16 | 16 | 16 | 16 | 0 | 0 | 0 | 0 |
| BSA | none | 87 | 38 | 69 | 35 | 9 | 3 | 9 | 0 |
| BSA | α v | 20 | 20 | 12 | 12 | 8 | 8 | 0 | 0 |
| Serum | none | 268 | 145 | 8 | 0 | 40 | 2 | 220 | 143 |

[VN, vitronectin; FN, fibronectin; EHS, EHS matrix; TSP, thrombospondin; BSA, bovine serum albumin; α v, C8F12 rat anti-mouse α v integrin subunit (200 μ g/ml); β 1, R48 rabbit anti-mouse β 1 integrin subunit (650 μ g/ml); IG, rat IgG (200 μ g/ml)]

Appendix 4.2 Effect of antibody concentration on embryo outgrowth inhibition

| Substrate | Antibody concentration | Total number of embryos | % outgrowing | |
|------------|---------------------------|----------------------------|--------------|-------|
| | | | Day 3 | Day 4 |
| Serum | none | 26 | 65 | 88 |
| Serum | 20µg/ml anti-αv | 26 | 54 | 85 |
| Serum | 40µg/ml anti-αv | 25 | 44 | 72 |
| Serum | 100µg/ml anti-αv | 30 | 40 | 53 |
| Serum | 200µg/ml anti-αv | 29 | 28 | 0 |
| Serum | 400µg/ml anti-αv | 20 | 0 | 0 |
| EHS matrix | none | 25 | 28 | 68 |
| EHS matrix | 650µg/ml anti-β1 | 20 | 0 | 0 |
| EHS matrix | 1300µg/ml anti-β1 | 19 | 0 | 0 |

Appendix 4.3 Substrate dependent effects of culture condition

| Comparison of outgrowth success | χ^2 test p value | Level of significance |
|---|---|----------------------------------|
| day 3 vitronectin-anti- α v vs. day 3 fibronectin-anti- α v | = 0.1848 | NS |
| day 3 vitronectin-anti- β 1 vs. day 3 fibronectin-anti- β 1 | = 0.0983 | NS |
| day 3 vitronectin-anti- α v vs. day 4 EHS matrix-anti- β 1 | = 0.0004 | *** |
| day 3 vitronectin-anti- β 1 vs. day 4 EHS matrix-anti- α v | = 0.6330 | NS |
| day 4 vitronectin-anti- α v vs. day 4 fibronectin-anti- α v | = 0.0004 | *** |
| day 4 vitronectin-anti- β 1 vs. day 4 fibronectin-anti- β 1 | = 0.4997 | NS |
| day 3 fibronectin-anti- α v vs. day 4 EHS matrix-anti- β 1 | \leq 0.0001 | *** |
| day 3 fibronectin-anti- β 1 vs. day 4 EHS matrix-anti- α v | = 0.5167 | NS |

[NS, non-significant]

Appendix 4.4 Effect of culture condition compared to negative control substrate

| Comparison of outgrowth success | χ^2 test p value | Level of significance |
|--|---|----------------------------------|
| <i>Day 3 of outgrowth</i> | | |
| BSA-control vs. vitronectin-anti- α v | = 0.2870 | NS |
| BSA-control vs. vitronectin-anti- β 1 | ≤ 0.0001 | *** |
| BSA-control vs. vitronectin-anti- α v+anti- β 1 | = 0.7704 | NS |
| BSA-control vs. fibronectin-anti- α v | = 0.0069 | * |
| BSA-control vs. fibronectin-anti- β 1 | ≤ 0.0001 | *** |
| BSA-control vs. fibronectin-anti- α v+anti- β 1 | = 0.9373 | NS |
| BSA-control vs. EHS matrix-anti- α v | = 0.2514 | NS |
| BSA-control vs. EHS matrix-anti- β 1 | = 0.0085 | * |
| BSA-control vs. EHS matrix-anti- α v+anti- β 1 | = 0.0516 | NS |
| <i>Day 4 of outgrowth</i> | | |
| BSA-control vs. vitronectin-anti- α v | ≤ 0.0001 | *** |
| BSA-control vs. vitronectin-anti- β 1 | ≤ 0.0001 | *** |
| BSA-control vs. vitronectin-anti- α v+anti- β 1 | = 0.0051 | * |
| BSA-control vs. fibronectin-anti- α v | = 0.0258 | NS |
| BSA-control vs. fibronectin-anti- β 1 | ≤ 0.0001 | *** |
| BSA-control vs. fibronectin-anti- α v+anti- β 1 | = 0.3870 | NS |
| BSA-control vs. EHS matrix-anti- α v | ≤ 0.0001 | *** |
| BSA-control vs. EHS matrix-anti- β 1 | E | NS |
| BSA-control vs. EHS matrix-anti- α v+anti- β 1 | E | NS |
| [NS, non-significant; E, no successes] | | |

Appendix 5 Restriction enzyme site map of known partial mouse $\beta 5$ sequence

Fragment MS β 5-1:

```

      N M
      l SsS      B
      a fpa B B c
      I aAc a m e
      I NI I n g f
      I III I I I
      atgccgcgggtgcccgcgaccctctacgccg
30 -----+-----+-----+-----+ 60
      tacggcgcccacggcgcgctgggagatgcggc

      B
      s
      s
      H
      I
      I
      C
      N v SB
      d i pf
      e R ea
      I I II

      tctgctcgggctctgcgcgctcgttccgcgcctcgcagggtcaacatatgcactagtgg
61 -----+-----+-----+-----+ 120
      agacgagcccagacgcgcgagcaaggcgaggagctcccgagttgtatacgtgatcacc

      D
      B r
      s a
      s I
      S I
      I I
      S
      a
      A Tu D
      l a3 p
      w qA n
      I II I

      B
      s
      T i
      a H
      q K
      I A
      I I

      aagtgccacctcgtgtgaanaatgcctgtcgcgatccacccaaaatgtgccnggtgctccaa
121 -----+-----+-----+-----+ 180
      ttcacggtggagcacacttnttacggacagctaggtgggttttacacggnccacgaggtt

      S
      Aa
      vu B
      a9 c
      I6 c
      II I
      /
      MT
      as
      ep
      I4
      I5
      II
      /

      agagtatttnggcnatccacggtccatcncctctcgggtgtgacctgaaggcanacctcac
181 -----+-----+-----+-----+ 240
      tctcataaanccgntaggtgccaggtagnggagagccacactggacttcogtntggagtg

      E
      c
      o
      5
      7
      I
      S
      m
      a
      I

      ccggaatggctgtgaagggtganattganantccagcc
241 -----+-----+-----+-----+ 278
      ggcccttaccgacacttccactntaactntnaggtcgg

```

Enzymes that cut:

AlwI, Avall, BanI, BclI, BceII, BfaI, BmgI, BseRI, BsiHKAI, BssHII, BssSI,
CviRI, DpnI, DraIII, Eco57I, MaeIII, MspAII, NdeI, NlaIII, SacII, Sau96I
Sau3AI, SfaNI, SmaI, SpeI, TaqI, TaqII, Tsp45I

Fragment MS β 5-2:

```

      B
      s H
      p i
      1 n
BB    CBS    B 2 d
ss    osc    m 8 I
ap    Rar    g 6 I
WE    IJF    I I I
II    III    I I I
/
cttggagaacatccggagcctgggcaccaagcttngcaggaaatgaggaagctcactag
488 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 547
gaacctcttgttaggcctcgacccgtggttcgaacncgtccttactccttcgagtgatc

      B
      p
      A uD
      c ld
      i 0e
      I II
      /
taacttcgcttaggtttcgggtcttttgttgacaaggacatctctctttctcctacac
548 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 607
attgaaggcgaatccaaagcccagaaaacaactgttcctgtagagaggaaagaggatgtg

      C      B      B
      j      c      s H
      e      e      m g
      I      f      F a
      I      I      I I
ggcaccgagataccagaccaatccgtgtattggttacaagttattccccaactgcgtccc
608 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 667
ccgtggctctatggtctggttaggcacataaccaatgttcaataaggggttgacgcaggg

      T
      t
      hH
      li SAT P
      ln aca l
      lf lcq e
      II III I
      / /
ctnctttcgggtccggcatctgctgcctctcacagacagagtcgacagcttcaa
668 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 721
gangaaagccaaggccgtagacgacggagagtgctgtctcagctgtcgaagtt

```

Enzymes that cut:

Accl, Acil, ApaBI, BbvI, Bcefl, Bfal, BmgI, Bpu10I, BsaJI, BsaWI, BsmFI,
BsoFI, Bsp1286I, BspEI, Cjel, Ddel, EcoRII, Hgal, HindIII, HinfI, MwoI,
PleI, Sall, ScrFI, SfaNI, SpeI, TaqI, TseI, Tth111I

Fragment MSβ5-3:

```

                                B
                                p
                                u
                                1
                                0
                                I
                                /
                                NS
                                sp
                                ph
                                II
                                /
2054  -----+-----+-----+-----+-----+-----+-----+-----+ 2112
      tgaccaggaggtgtgtgtttgcttctacaaaactgctaaggcatgcgttatgatgttcag
      actggtcctccgacacgaaacgaagatgttttgacgattccgtacgcaataactacaagtc

                                M
                                m
                                e
                                I
                                P
                                f
                                l
                                M
                                I
2113  -----+-----+-----+-----+-----+-----+-----+ 2172
      ctacacagaactgcccaatgggaggtccaacttgacggtcctccgggagccagaatgtgg
      gatgtgtcttgacgggttacccctccaggttgaactgccaggaggccctcggtcttacacc

                                B
                                s
                                t
                                X
                                I
                                S
                                f
                                a
                                N
                                I
2173  -----+-----+-----+-----+-----+-----+-----+ 2232
      aagtgcaccaatgccatgaccatcctgctgtggttggcagcatcctcctgattgg
      ttcacgggggttacggtactggttaggacgaccgacaccaaccgtcgtagggactaacc

                                E M
                                a s
                                e c
                                I I
                                H
                                p
                                h
                                I
                                B
                                s
                                i
                                E
                                I
2233  -----+-----+-----+-----+-----+-----+-----+ 2292
      gatggcactcctggccatctggaagctgctogtcaccatccacgaccgccgagagtttgc
      ctaccgtgaggaccggtagaccttcgacgagcagtggttaggtgctggcggtctctaaacg

                                E
                                c
                                o
                                4 H
                                7 a
                                I e
                                I I
                                I I
                                E
                                c
                                o
                                O
                                1
                                0
                                9
                                I
                                BB
                                Aas
                                pnc
                                aIG
                                III
                                //
2293  -----+-----+-----+-----+-----+-----+-----+ 2352
      caagttccaaagtgagcgctccagggcccggttatgaaatggcctcaaaccctgtacag
      gttcaagggtttcactcgcgaggtcccgggcaatactttaccggagtttgggggacatgtc

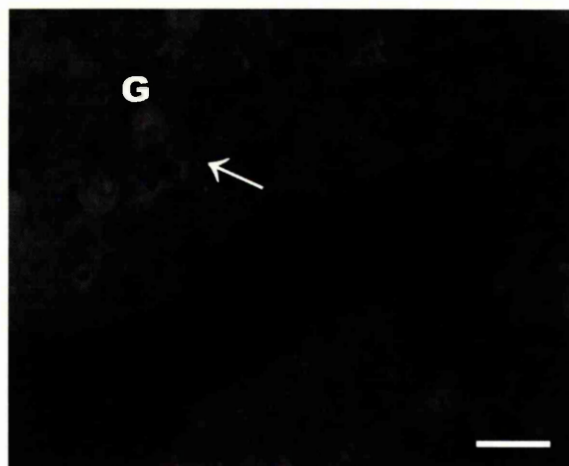
                                T
                                a
                                q
                                I
2353  -----+-----+-----+-----+-----+-----+-----+ 2412
      aaagcccatctccacacacactgtcgatttcgcnttcaacaaatcctacaatggctcagt
      tttcgggtagaggtgtgtgtgacagctaaagcgnaagttgttttaggatgttacccagtc

      ggactgaggctcc
2413  -----+----- 2426
      cctgactccgagg

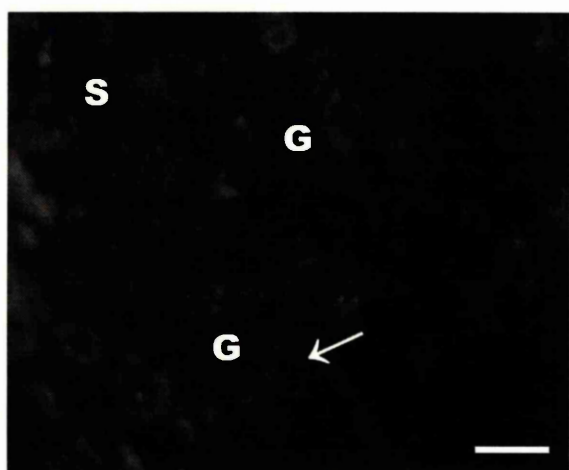
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Enzymes that cut:

Apal, BanII, Bpu10I, BscGI, BsiEI, BstXI, EaeI, Eco47III, EcoO109I,
EcoRI, HaeII, HphI, MmeI, MscI, NspI, PfiMI, SfaNI, SphI, TaqI



NEGATIVE CONTROL
Mid-secretory phase endometrium



NEGATIVE CONTROL
First trimester decidual glands



NEGATIVE CONTROL
First trimester decidual vessel

Appendix 6. Immunolocalisation of integrins in human endometrium

Negative control human endometrium at implantation and during early pregnancy
Pre-immune rabbit serum in place of anti-integrin primary antibody, see 3.2.2.1.

G, gland; **S**, stroma; **V**, blood vessel; **→** basal surface; scale bar = 20μm

PUBLISHED WORK

Integrins β_5 , β_3 and α_v are apically distributed in endometrial epithelium

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Several adhesion molecules have been shown to occur at the surface of endometrial cells. One of these is the integrin α_v subunit which associates with various β chains including β_5 . We demonstrate the presence of integrin β_5 polypeptide in human endometrial epithelial cells throughout the menstrual cycle using immunocytochemistry with monospecific antibodies, and at the mRNA level by thermal amplification from endometrial cDNA. Integrin β_5 is also found in a population of bone marrow-derived cells. A notable feature of the distribution of the β_5 subunit in the glandular and luminal epithelium is its apical localization, which may suggest an involvement in implantation. However, no evidence was found for regulated expression of epithelial β_5 . In mouse, the β_5 subunit is found at both the apical and basal surface of epithelial cells and expression is essentially oestrous cycle-independent. Comparisons are made in both species with the distribution of the α_v and β_3 subunits which also localize to the apical epithelium.

Key words: cell surface/decidua/embryo implantation/endometrium/integrin

Introduction

The endometrial epithelial cell surface is the first point of stable contact between the blastocyst and maternal tissue. Receptivity to implantation appears to be controlled by the endometrial luminal epithelium (Glasser and Mulholland, 1993; Tabibzadeh and Babaknia, 1995). Thus it is important to examine the cell surface composition of endometrial epithelium; the presence of adhesion and anti-adhesion molecules may indicate a function in intercellular interaction in early pregnancy. We and others have demonstrated the presence of integrins (Lessey *et al.*, 1992; Tabibzadeh, 1992; Klentzeris *et al.*, 1993; Aplin *et al.*, 1994; Albers *et al.*, 1995; reviewed in Bronson and Fusi, 1996) and other adhesion molecules (Lindenberg *et al.*, 1988; Kimber *et al.*, 1993; Behzad *et al.*, 1994; Fukuda *et al.*, 1995) in endometrial epithelium in human and mouse. MUC1, an apically disposed epithelial cell surface molecule with anti-adhesion properties, is expressed in the endometrium in both these species (Braga and Gendler, 1993; Hey *et al.*, 1994, 1995). Integrin β_3 , which associates with α_v to form a multifunctional receptor for several extracellular arginine–glycine–aspartic acid (RGD)-containing ligands including fibronectin, vitronectin, fibrinogen and osteopontin (Hynes, 1992; Felding-Habermann and Cheresch, 1993), shows an interesting pattern of regulation in human endometrial glandular epithelium, with onset of expression at approximately day 19 of the cycle (Lessey *et al.*, 1992). Since the α_v subunit is present in the same cells, but appears not to share this pattern of regulation (Lessey *et al.*, 1992), it appears likely that other β -subunits capable of association with α_v may also be expressed. In this study we have examined the expression

of integrin β_5 , which associates with α_v to form an RGD-dependent receptor for fibronectin, vitronectin or osteopontin (Felding-Habermann and Cheresch, 1993; Liaw *et al.*, 1995). We show that this integrin is present in the luminal epithelium in both human and mouse endometrium at the time of implantation.

Materials and methods

Animals

MF1 mice (Harlan OLAC, Bicester, UK) were kept in a controlled environment with a cyclic photoperiod of 12 h light followed by 12 h darkness and provided with food and water *ad libitum*. Ovariectomized mice were rested for 10 days then injected with vehicle (corn oil) or 100 ng oestradiol benzoate daily for 2 days followed by 2 days' rest. Oestrogen-primed animals were then divided into three groups. The first group received 100 ng oestradiol benzoate daily for 4 further days. The second group received 500 ng progesterone daily for 4 further days. The third group received 500 ng progesterone daily for 3 days, then 500 ng progesterone with 10 ng oestrogen on the fourth day. They were killed by cervical dislocation 16 or 18 h after the last injection. Pregnant uterine horns were taken from naturally-mated MF1 female mice. The animals were paired with MF1 male mice and left overnight. Mating was assumed to occur at approximately midnight. The following morning successful mating was confirmed by the presence of a vaginal plug. This day was designated as day 1 of pregnancy.

The uterine horns were trimmed of fat, removed and placed in Hank's Balanced Salt Solution (HBSS; Gibco BRL, Paisley, UK). Tissue from three different animals was used to study each day of pregnancy, the oestrous cycle and there were also three different

animals in each ovariectomy treatment group. No significant inter-animal variation was seen in tissue at the same stage.

Tissue collection and storage

Human endometrial tissue was obtained at routine dilatation and curettage. First trimester decidua and placenta were obtained at termination of pregnancy. In all cases, patient consent and local ethical committee approval was obtained. Mouse uterine horns taken for immunolocalization studies were cut transversely into 0.5 cm pieces, embedded in OCT compound (R.Lamb, Cambridge, UK) and snap-frozen in liquid nitrogen. Human endometrial curettings were either treated similarly or fixed in Bouin's solution and wax-embedded. Frozen tissue was stored at -80°C until required. Transverse sections (6 μm) were cut using a Reichert-Jung cryostat. Sections were air dried and stored at -20°C until use. Human tissue was dated by histological examination using the criteria of Noyes *et al.* (1950), or in relation to the luteinizing hormone (LH) peak. Neoplasia and infection were excluded in all cases. A total of 18 frozen specimens (nine proliferative and nine secretory phase) of human tissue were studied. Integrin β_5 was also examined in a further 20 specimens of human tissue that had been routinely fixed in Bouin's solution then processed into wax. These included four from each of the proliferative, early secretory, mid-secretory, mid-late secretory and late secretory phases.

Immunostaining of endometrium

Cryosections were fixed in acetone (-15°C) for 10 min, then brought to room temperature. After thorough washing with phosphate-buffered saline (PBS), sections were blocked using normal goat serum (1:20) incubated with primary antibody for 1 h in a humid chamber. After further washes, sections were incubated for 1 h with biotinylated secondary antibody (anti-rabbit or anti-mouse; Dako, High Wycombe, UK) diluted to 1:50 in PBS. Once washed, sections were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Dako) diluted to 1:50 in PBS, for 1 h. Sections were again thoroughly washed in PBS. Controls using an inappropriate primary antibody (monoclonal), non-immune rabbit serum or PBS in place of the primary antibody, secondary antibody or FITC-streptavidin were performed and were negative in all cases. In some cases, the primary antibody was visualized with FITC-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulins (adsorbed with human and bovine immunoglobulins; Dako) diluted 1:50 in PBS. Results were identical with the two-step and three-step methods. Stained sections were mounted in Immu-Mount (Shandon, Warrington, U.K.) or in 25 mg/ml 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Sigma, Poole, UK) in 90% glycerol, 10% PBS and photographed with Ilford HP5 film rated at 1000 ASA.

Routinely-processed tissue was dewaxed in xylene, endogenous peroxidase activity quenched using 1/100 (v/v) hydrogen peroxide in absolute alcohol (30 min), then sections were treated with 70% alcohol, distilled water (three changes) and then tris-buffered saline (TBS). Sections were treated with 0.1% trypsin for 15 min at 37°C . Non-specific binding was blocked using Dako protein block [10 min room temperature (RT)] followed by blocking of non-specific biotin/avidin binding (Blocking Kit, Vector, Peterborough, UK). First antibody was applied for 1 h RT, followed by three 5 min washes in TBS. Incubation with second antibody followed [biotinylated anti-rabbit (Dako), 1:200 in TBS, 1 h, RT]. After three further washes in TBS, Vector ABC reagent was added (30 min, RT) followed by washing and colour development in DAB (3,3'-diaminobenzidine) with nickel enhancer. Experiments were also carried out using microwave pretreatment of sections; the results were essentially the same. The panel of antibodies used in the study is given in Table I.

Isolation of decidual stromal cells

Parietal decidua was isolated from first trimester terminations and digested with collagenase/hyaluronidase to isolate decidual stromal cells as previously described (Vicovac *et al.*, 1994). Cells were cultured in monolayer in Dulbecco's Modified Eagle's Medium (DMEM)/10% fetal calf serum. They were fixed in acetone in preparation for immunostaining.

Isolation of endometrial gland cells

The procedure has been described previously (Hey *et al.*, 1994). Endometrial curettings were washed in PBS, finely chopped with a scalpel and digested with a mixture of 1 mg/ml crude collagenase (Sigma; type I), 1 mg/ml crude hyaluronidase (Sigma) and 0.2 mg/ml DNase (Sigma). Glandular fragments were purified by low speed centrifugation (800 g, 2 min) and differential filtration. Undigested tissue fragments were removed by filtration through a 400 μm polyester mesh. Glandular fragments were trapped on a 30 μm mesh and single cells were removed by washing the filter with PBS. The filter was inverted and the purified glandular fragments were collected by back-flushing and either snap-frozen in liquid nitrogen or used immediately to produce total RNA. The cell purity in the fragments was assessed by cytokeratin 8/18 (monoclonal antibody CAM5.2, Becton-Dickinson, High Wycombe, UK) staining after outgrowth *in vitro*, and found to be $>95\%$.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from tissue or isolated glandular fragments by acid guanidinium thiocyanate-phenol-chloroform extraction as described previously (Hey *et al.*, 1994). RNA ($\sim 50 \mu\text{g}$) was obtained from glandular fragments from each specimen. Reverse transcription of the mRNA was carried out using the Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV) kit (Gibco BRL). The reaction mixture contained Tris-HCl (50 mM), KCl (75 mM), MgCl_2 (3 mM), dithiothreitol (10 mM), 1 mM of each dATP, dTTP, dCTP, dGTP, 1 IU RNAGuard (Pharmacia, St. Albans, UK), 1.5 mg poly dT, 200 IU reverse transcriptase and 10 μg of RNA in a total volume of 20 μl . The reaction was carried out by incubation at 37°C , 65 min and terminated by heating to 80°C , 10 min. 2 ml of the reaction mixture was used as a template for PCR. PCR primers were as follows (residue numbers from McLean *et al.*, 1990):

UB5 (2188): 5'TGGAAACACCCCCAACGCCATGACCAT3'

LB5 (2392): 5'AACTTGTTGAAGGTGAAGTCCACA3'

PCR reactions were carried out in a total volume of 20 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl_2 , 0.2 mM each dATP, dTTP, dCTP and dGTP plus 0.5 units of *Taq* polymerase enzyme (Promega, Southampton, UK). Amplifications used an initial denaturation step of 95°C , 5 min and 35 cycles each of 1 min at 95°C , 1 min at 60°C . An extension step of heating to 72°C for 5 min was included at the end of the programme. The products were electrophoresed in 1.0% agarose gels containing 0.5 g/ml ethidium bromide. Gland cell preparations were also used to amplify a sequence spanning the major site of mRNA splicing in CD44 as described previously (Behzad *et al.*, 1994) again with 35 cycles of PCR. The preparations contained long isoforms (CD44E), but little or no short isoform (CD44H). Since CD44H is the major stromal and immune cell form of CD44, we conclude that there was very little non-epithelial mRNA in the isolates used for integrin RT-PCR.

Results

Integrin subunit expression in mouse endometrium

Sections of mouse uterus from all stages of the oestrous cycle and the first 6 days of pregnancy were stained by

Table I. Panel of antibodies used

| Subunit specificity | Name (clone) | Source | Species specificity | Host | Isotype specificity | Epitope(s) | Working dilution |
|---------------------|----------------|------------------------|---------------------|--------|----------------------|---|------------------|
| α_v | AB1930 | Chemicon International | Human | Rabbit | Polyclonal antiserum | Cytoplasmic domain of synthetic peptide | 1:40–1:80 |
| α_v | MAB1978 | Chemicon International | Human | Mouse | Monoclonal IgG | Not indicated | 1:1000 |
| β_3 | AB1932 | Chemicon International | Human | Rabbit | Polyclonal antiserum | Purified protein from human platelets | 1:40–1:100 |
| β_5 | AB1926 | Chemicon International | Human | Rabbit | Polyclonal antiserum | Cytoplasmic domain of synthetic peptide | 1:40–1:80 |
| $\alpha_v\beta_5$ | MAB1961 (P1F6) | Chemicon International | Human | Mouse | Monoclonal IgG1 | Ligand binding domain (?) | 1:500 |

Ig = immunoglobulin.

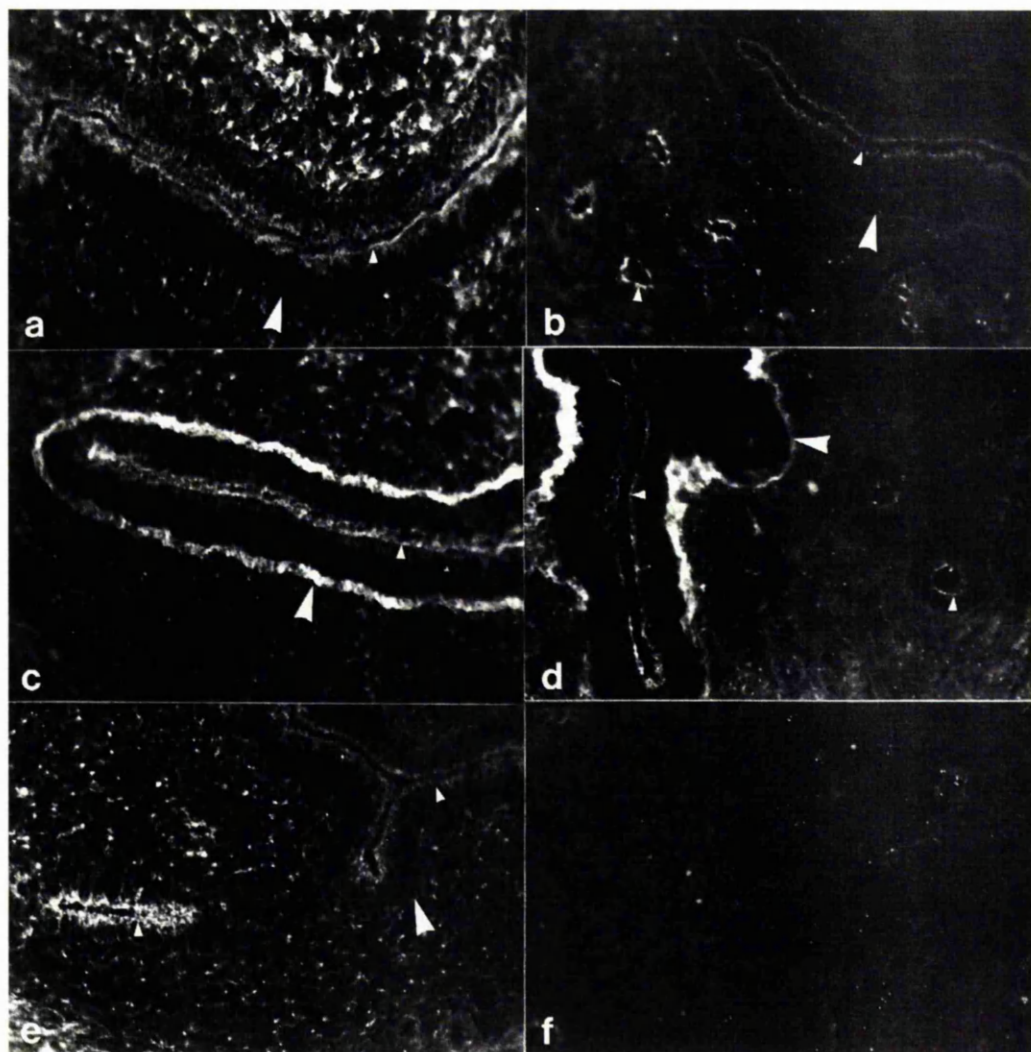


Figure 1. Immunofluorescence of integrin subunits β_5 (c, d), β_3 (b) and α_v (a, e) in mouse pregnant uterus and uterus from ovariectomized animals stimulated with exogenous steroid. (a–c) Pregnancy day 5; (d–f) ovariectomized animals treated with (d) vehicle alone, (e) progesterone, (f) oestradiol. Note prominent reactivity of the β_5 subunit in the basal and apical luminal epithelium, while α_v and β_3 show a more apical distribution. Some stromal reactivity is present in all three subunits. The distribution of each subunit was very similar in pregnant, cycling and ovariectomized mice with or without hormone treatment. Representative examples are shown (original magnification $\times 390$); (f) is a negative control. Basal epithelial surfaces are indicated by large arrowheads. Apical epithelial surfaces (luminal and glandular) are indicated by small arrowheads (a–e).

immunofluorescence to determine the distribution of the β_v , β_3 and β_5 integrin subunits (Figure 1). At all stages of pregnancy and the oestrous cycle examined, immunoreactivity

for the β_5 (Figure 1c,d) and β_3 (Figure 1b) subunits was seen in the apical surface of both glandular and luminal epithelial cells. Slight increases in β_3 staining intensity were

Table II. Results of mouse uterus staining

| | Apical luminal epithelium | Basal luminal epithelium | Apical glandular epithelium | Basal glandular epithelium | Lateral epithelium | Stroma |
|--|---------------------------------|--------------------------------|-----------------------------------|----------------------------------|-----------------------|--------|
| α_v | | | | | | |
| Pro-oestrous | + | + | + | (+) | + | + |
| Oestrous | + | + | ++ (D) | (+) ++ (D) | + | + |
| Metooestrous | ++ (D) | + | ++ (D) | + | + | + |
| Dioestrous | ++ (D) | (+) ++ (D) | ++ (D) | (+) ++ (D) | (+) | + |
| Ovariect | + | (+) | + | (+) | + | + |
| Ovariect + oestrogen | + | + | + | (+) | + | + |
| Ovariect + progesterone | + | (+) | + | (+) | + | + |
| Ovariect + oestrogen + progesterone | + | (+) | + | (+) | (+) | + |
| Day 1 | + | (+) | + | (+) | | + |
| Day 2 | ++ (D) | (+) ++ (D) | + | (+) | (+) | + |
| Day 3 | + | (+) | + | (+) (D) | (+) ++ (D) | ++ (D) |
| Day 4 | + | (+) | + | (+) | (+) | + |
| Day 5 | + | (+) | + | (+) | + | + |
| Day 6 | + | (+) | + | (+) | + | + |
| β_3 | | | | | | |
| Pro-oestrous | + | + | + | + | + | + |
| Oestrous | + OR ++ | + | + OR ++ | (+) | (+) | + |
| Metooestrous | ++ | + | ++ | + | (+) | (+) |
| Dioestrous | + | + | + | (+) | (+) | + |
| Ovariect | + | + | + | (+) | (+) | (+) |
| Ovariect + oestrogen | + | + | + | + | + | (+) |
| Ovariect + progesterone | ++ | + | ++ | (+) | + | + |
| Ovariect + oestrogen + progesterone | ++ | + | ++ | (+) | + | + |
| Day 1 | + | + | + | (+) | | + |
| Day 2 | + | (+) | + | (+) | (+) | + |
| Day 3 | + | (+) | + | (+) | (+) | + |
| Day 4 | + OR ++ | + | + OR ++ | (+) | (+) | (+) |
| Day 5 | ++ | (+) | + | (+) | | + |
| Day 6 | ++ | + | + | (+) | | + |
| β_5 | | | | | | |
| Pro-oestrous | + | ++ | + | + | (+) | + |
| Oestrous | + | ++ | + | (+) | + | + |
| Post-oestrous | + | ++ | + | + | + | + |
| Dioestrous | + | ++ | + | (+) | (+) | + |
| Ovariect | + | ++ | + | (+) | (+) | + |
| Ovariect + oestrogen | + | ++ | + | (+) | (+) | + |
| Ovariect + progesterone | + | ++ | + | (+) | (+) | + |
| Ovariect + oestrogen + progesterone | + | ++ | + | (+) | + | + |
| Day 1 | + | ++ | + | (+) | | + |
| Day 2 | + | ++ | + | (+) | (+) | + |
| Day 3 | + | ++ | + | (+) | (+) | + |
| Day 4 | + | ++ | + | (+) | (+) | + |
| Day 5 | + | ++ | + | (+) | | + |
| Day 6 | + | ++ | + | (+) | | + |

Staining intensity graded as: (+) = weak; + = moderate; ++ = high.
D = denotes discrete patches of differently stained cells.

observed in the luminal epithelium on days 4–6 of pregnancy. β_3 and β_5 integrin subunits were also weakly detected in the lateral surface domains of epithelial cells. Integrin β_5 was strongly expressed at the basal luminal epithelial cell surface. However, it was much more weakly detected at the basal surface of glandular epithelial cells (Figure 1d). Integrin β_3 was detected at moderate intensity in the basal surface domains of both epithelial populations. There were no striking changes in staining intensity at any stage for β_5 .

Staining for the α_v subunit was more heterogeneous. A few very brightly stained patches of epithelium were seen at all stages of the oestrous cycle and on days 2 and 3 of

pregnancy, but not at other stages. The entire surface and cytoplasm of the epithelial cells was often stained in these areas. In most other areas the apical region of luminal (Figure 1a) and glandular epithelium showed weak to moderate staining at all stages. The basal epithelial surface was poorly stained with antibody to α_v .

All three subunits were weakly detectable throughout the endometrial stroma at all stages. α_v expression in stroma had a characteristic punctate pattern (Figure 1e) and appeared higher on days 5 and 6 of pregnancy (Figure 1a).

In order to confirm the lack of major steroidal effects on integrin expression, ovariectomized mice were treated

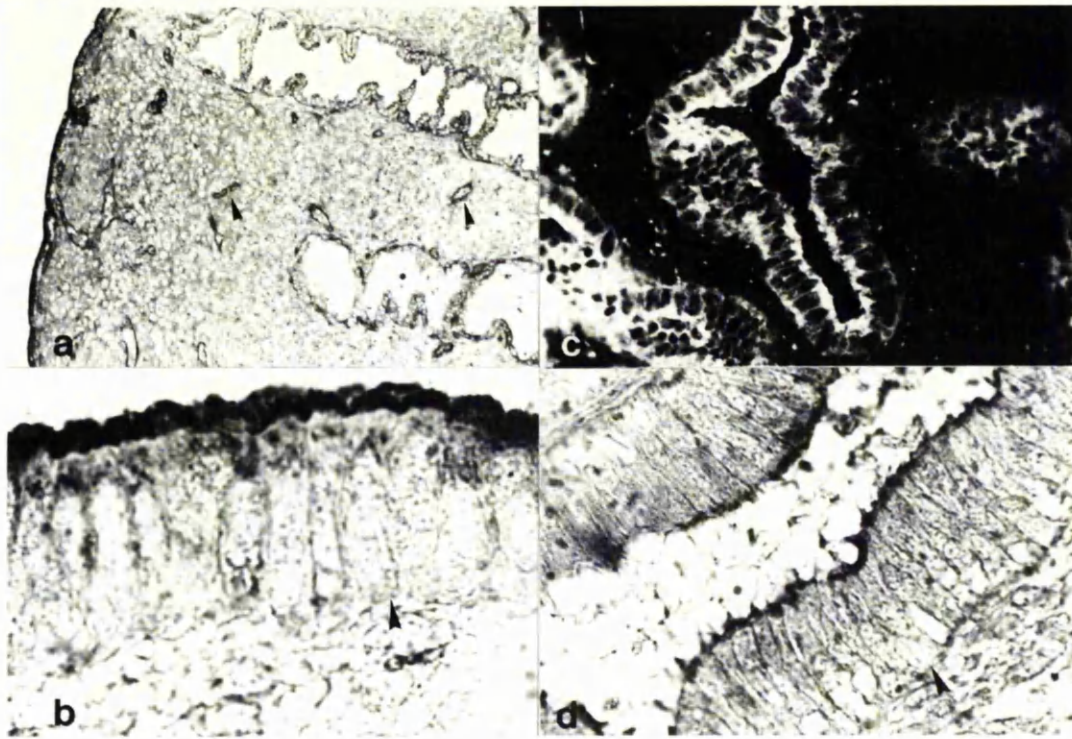


Figure 2. Immunolocalization of integrin subunits β_5 (a, b, d) and α_v (c) in human endometrium using ABC peroxidase (a, b, d) or indirect immunofluorescence (b, c). Note the intense apical reactivity of the β_5 subunit both in the luminal (a, b) and glandular cells (a, d). β_5 can also be seen in the stroma and blood vessels (arrowheads in a). α_v is evident in all cell surface domains in the epithelium as well as a weaker reactivity in stromal cells (c). Little variation is observed through the menstrual cycle. Basal epithelial surfaces are indicated by arrowheads in b and d. Original magnifications: (a) $\times 80$, (b) $\times 390$, (c) $\times 1170$, (d) $\times 780$.

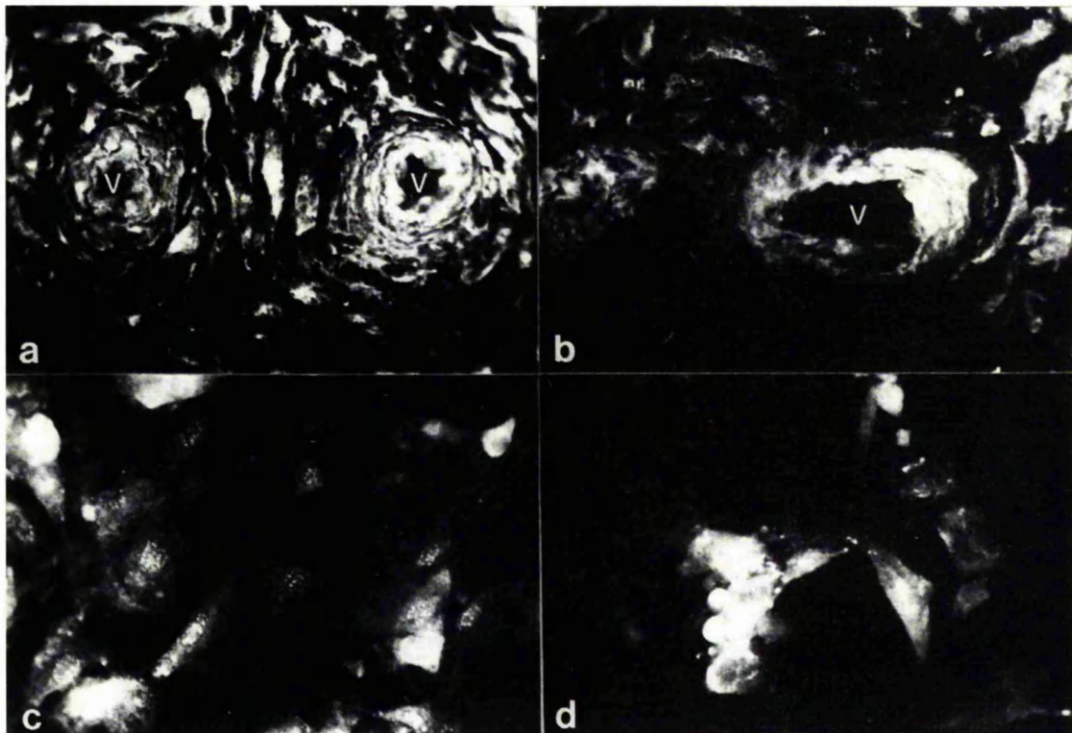


Figure 3. Immunofluorescence of integrin subunits α_v (a, c) and β_5 (b, d) in human first trimester decidua. Both are evident in stromal cells and the walls of blood vessels. In-vitro variation is observed in the staining intensity. Punctate reactivity, apparently nuclear, is observed in cultured cells with antibody to α_v (c). Staining with anti- β_5 is more diffuse (d) (original magnification $\times 390$).

with various regimes of exogenous steroids and the distribution of the various subunits in the uterus was examined. Treatment with vehicle alone was compared with

oestrogen alone or in combination with progesterone as described in the methods section. A slight increase in β_3 immunoreactivity was noted in ovariectomized mice under

Table III. Results of human endometrial immunostaining for integrin β_5

| Phase | Luminal epithelium | Glandular epithelium | Stroma | Lymphocytes |
|-----------------------|--------------------|----------------------|----------------|-------------|
| Proliferative | + / + + | + / + + | + / + + (b.v.) | ++ |
| Early secretory | ++ | ++ | + / + + | ++ |
| Mid-secretory | ++ | ++ + / + + | ++ | |
| Mid-to-late secretory | ++ | + / + + | + | ++ |
| Late secretory | ++ | ++ | + (b.v.) | ++ |

Staining intensity: + = moderate; ++ = high
b.v. = blood vessels.

the influence of progesterone. Otherwise subunit distribution was identical in hormone-treated and untreated animals (Figure 1; Table II).

The myometrium was observed to express α_v , β_3 and β_5 at all stages. Some blood vessels of the endometrium stained weakly for α_v and weakly to moderately for β_3 and β_5 .

Integrin expression in human endometrium and decidua

A wider repertoire of antibodies was available to human subunits (Table I). These included monoclonal antibodies that recognize the heterodimeric complex $\alpha_v\beta_5$. The subunits (Figure 2) and $\alpha_v\beta_5$ integrin dimer (not shown) are expressed by the luminal and glandular epithelial cells of human endometrium (Figure 2) and first trimester decidua (Figure 3). Staining with antibodies to the α_v (Figure 2b) subunit was present in the apical, lateral and basal epithelial cell surfaces with weaker stromal reactivity. The distribution was not observed to alter significantly with cycle phase. In contrast, the β_5 subunit was detected in both glandular and luminal epithelial cells with a dominant apical distribution (Figure 2a,c,d). It was also evident in blood vessels (Figure 2a). There was no significant change in staining intensity or distribution in the epithelium from the proliferative to the secretory phase (Table III).

In decidua, immunoreactivity for the α_v subunit was associated with stromal and vascular cells (Figure 3a). Variable levels of reactivity with anti- β_5 were observed in the resident decidual stromal and vascular cells (Figure 3b). Similar observations were made in cultured decidual stromal cells (Figure 3c,d).

RT-PCR

Evidence that mRNA encoding integrin β_5 subunit is present in human glandular epithelial cells was obtained by RT-PCR. Gland fragments were prepared and used to produce cDNA. A product of the predicted size of 228 bp was readily detectable after PCR using cDNA produced from total endometrial RNA in either the proliferative or secretory phases (Figure 4).

Discussion

Previous studies have indicated that human endometrial epithelial cells express integrins of the β_1 family at the basolateral surface domain in both glandular and luminal compartments (Lessey *et al.*, 1992; Tabibzadeh, 1992; Klentzeris *et al.*, 1993; Albers *et al.*, 1995). Some of the α subunits that associate

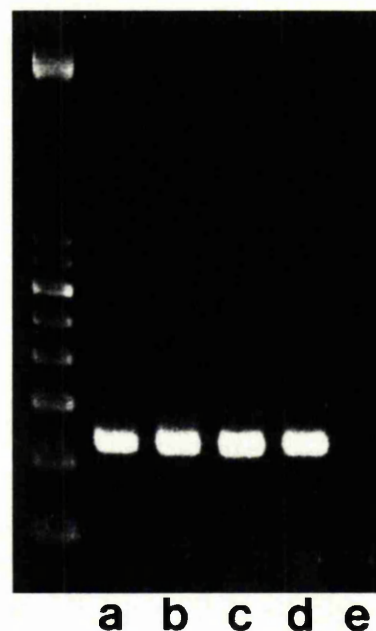


Figure 4. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of mRNA encoding integrin β_5 from isolated human glandular epithelial cells in proliferative (a, b) or secretory phase (c, d). A single specific product is observed in both cycle phases. (e) negative control. The left hand lane contains a 100 bp ladder.

with β_1 appear highly polarized (for example, α_1 and α_6 are confined to the basal or basolateral surface) while others are less so (for example, the α_2 subunit has been described in both apical and basolateral locations). Previous localization studies of the α_v and β_3 integrin subunits have suggested non-polarized expression in glandular epithelial cells, and absence of reactivity of β_3 in the luminal epithelium (Lessey *et al.*, 1992; Taskin *et al.*, 1994; Albers *et al.*, 1995). Thus it is of considerable interest that the β_5 subunit is strongly expressed in the apical epithelial surface of both glandular and luminal cells in human. β_5 is thought only to associate with one α subunit, α_v , which is present at the apical epithelial surface. In the mouse, α_v and β_5 are expressed in the apical epithelial compartment in both luminal and glandular cells. These data predict, but do not unequivocally prove, that the heterodimeric species $\alpha_v\beta_5$ is present in the apical epithelial cell surface. In the human its presence has been confirmed by recognition with a monoclonal antibody to the $\alpha_v\beta_5$ complex. Both in human and mouse, the distribution is essentially independent

of hormonal regulation. Thus $\alpha_v\beta_5$ is predicted in both species at the site of initial embryo attachment.

Integrin $\alpha_v\beta_5$ is not currently believed to mediate direct interaction with cell surface-associated ligands on mammalian cells. Its ligand repertoire is based on RGD motifs on extracellular glycoproteins including vitronectin, fibronectin and osteopontin (Felding-Habermann and Cheresch, 1993; Liaw *et al.*, 1995) although it has also been shown to interact with the Tat protein of human immunodeficiency virus (HIV) (Vogel *et al.*, 1993) and the penton base protein of adenovirus (Wickham *et al.*, 1992). Thus it is possible that the trophectodermal cells of implanting blastocysts may express a novel ligand for $\alpha_v\beta_5$. Alternatively, a model could be envisaged in which a bifunctional extracellular ligand present in the uterine fluid might 'bridge' between epithelial $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ and a receptor at the trophectodermal surface. There is no current evidence for the presence of vitronectin in the uterine cavity (Aplin *et al.*, 1988). Fibronectin has been described in association with the zona pellucida of human embryos (Turpeenniemi-Hujanen *et al.*, 1995). Other possible candidates are osteopontin and thrombospondin. Osteopontin is expressed by endometrial epithelial cells in the secretory phase of the cycle (Brown *et al.*, 1992), is bifunctional and acts as a ligand for both $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Thrombospondin is a ligand for $\alpha_v\beta_3$ and is expressed by trophoblast, glandular epithelium and decidua (O'Shea *et al.*, 1990; Corless *et al.*, 1992). Moreover, antibodies to thrombospondin block outgrowth of murine trophoblast on a thrombospondin substrate *in vitro* (O'Shea *et al.*, 1990). It is interesting in this context to note that both human and mouse blastocysts express integrins of the α_v family (Sutherland *et al.*, 1993; Campbell *et al.*, 1995; Stephens *et al.*, 1995; Yelian *et al.*, 1995). Indeed $\alpha_v\beta_3$ is expressed by murine trophoblast outgrowths on laminin and fibronectin (Sutherland *et al.*, 1993; Yelian *et al.*, 1995) and binding of fibronectin-coated beads to blastocysts is inhibited by antibodies to $\alpha_v\beta_3$ (Schultz and Armant, 1995). β_1 integrins are also present on the embryo; interestingly, inactivation of this subunit by gene targeting results in implantation failure (Fässler and Meyer, 1995; Stephens *et al.*, 1995). However, this failure appears not to be in attachment or epithelial penetration, but rather the subsequent penetration of the stromal compartment. Thus it remains an open possibility that α_v integrins play a role in the epithelial phase of embryo implantation.

The occurrence of strong basal epithelial β_5 immunoreactivity in mouse luminal epithelial cells (though not in the mouse glandular compartment and not in human epithelium) is surprising given the absence of prominent α_v expression in this location. Further studies will be required to examine the subunit association pattern and function of β_5 in this location. Given the differences in distribution between the α_v subunit and the two β -subunits included in the present study, it is also important to note that α_v associates with at least three other β subunits: β_1 , β_6 and β_8 . Both β_1 (see above) and β_6 (Beuss *et al.*, 1995) have been shown to be expressed in a basolateral distribution in endometrial epithelium. Other studies have indicated differences in the patterns and regulation of surface antigen expression between the luminal and glandular epithelial

cell populations in rodents (Martin and Finn, 1968; Kimber *et al.*, 1993).

The apparent lack of modulation in the expression of α_v and β_5 integrins in both mouse and human in response to either physiological or exogenous hormonal stimulation suggests that the maternal receptive period is not defined by the regulation of either of these subunits. However, it is unlikely that attachment is wholly dependent on one recognition event; indeed it is evident that non-integrin cell adhesion molecules are expressed at the apical epithelial cell surface at the time of implantation. Thus Fukuda *et al.* (1995) have suggested the possibility of intercellular adhesion mediated by a complex of the molecules trophinin and tastin associated with the cell surfaces of both blastocyst and endometrial epithelium. Furthermore, it may be that differential masking of constitutively expressed adhesion systems may allow modulation of adhesiveness. Thus it has been shown that MUC1, a cell surface-associated mucin that exhibits anti-adhesion properties, is expressed at the maternal cell surface (Braga and Gendler, 1993; Hey *et al.*, 1994, 1995). The mouse homologue Muc1 disappears at the time of implantation (Braga and Gendler, 1993), while human MUC1 is present in the mid-secretory phase and has been suggested to present a barrier to implantation (Aplin *et al.*, 1994, 1996).

Integrin β_3 has been reported to appear abruptly in human endometrial glands on day 20 of the cycle, coinciding with the onset of the receptive period (Lessey *et al.*, 1992). However, it appears to be absent from the luminal epithelium (Albers *et al.*, 1995) at least until a later stage of the luteal phase (Lessey *et al.*, 1992). $\alpha_v\beta_3$ expression by glandular epithelium continues in decidua of early pregnancy (Lessey *et al.*, 1992, 1994; Ruck *et al.*, 1994). Its expression is not significantly altered by administration of an emergency post-coital contraceptive containing 100 mg ethinyl oestrogen and 2 mg norgestrel (Taskin *et al.*, 1994) or by in-utero exposure to diethylstilbestrol (Castelbaum *et al.*, 1995). In the mouse oestrous cycle there is little change in the level or distribution of β_3 (or α_v or β_5), suggesting that expression is not significantly altered by changing concentrations and ratios of ovarian hormones. To investigate further the role of oestrogen and progesterone in the control of integrin expression, mice that had undergone ovariectomy and controlled hormone replacement were examined and again no variation of any of the subunits under study was observed as a function of exogenous oestrogen or progesterone. It is notable that, in the mouse, detectable levels of β_3 are present in the apical luminal epithelium and it certainly cannot be excluded that this integrin may play a role in implantation. If so, the absence of significant modulation by ovarian steroids would suggest that other factors must regulate its availability or state of activation in relation to the peri-implantation period.

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References

- Albers, A., Thie, M., Hohn, H.-P. and Denker, H.-W. (1995) Differential expression and localisation of integrins and CD44 in the membrane domains of human uterine epithelial cells during the menstrual cycle. *Acta Anat.*, **153**, 12–19.
- Aplin, J.D. (1996) Cell biology of human implantation. *Placenta*, **17**, 269–276.
- Aplin, J.D., Charlton, A.K. and Ayad, S. (1988) An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res.*, **253**, 235–240.
- Aplin, J.D., Seif, M.W., Graham, R.A. *et al.* (1994) The endometrial cell surface and implantation: expression of the polymorphic mucin MUC-1 and adhesion molecules during the endometrial cycle. *Ann. N.Y. Acad. Sci.*, **734**, 103–121.
- Behzad, F., Seif, M.W., Campbell, S. and Aplin, J.D. (1994) Expression of two isoforms of CD44 in human endometrium. *Biol. Reprod.*, **51**, 739–747.
- Beuss, J.M., Galo, J., Delisser, H.M. *et al.* (1995) Expression of the β_6 integrin subunit in developing neoplasia and tissue repair suggests a role in epithelial remodelling. *J. Cell Sci.*, **108**, 2241–2251.
- Braga, V.M.M. and Gendler, S.J. (1993) Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. *J. Cell. Sci.*, **105**, 397–405.
- Bronson, R. A. and Fusi, F.M. (1996) Integrins and human reproduction. *Mol. Hum. Reprod.*, **2**, 153–168.
- Brown, L.F., Berse, B., Van de Water, L. *et al.* (1992) Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. *Mol. Biol. Cell*, **3**, 1169–1180.
- Campbell, S., Swann, H.R., Seif, M.W. *et al.* (1995) Cell adhesion molecules on the oocyte and pre-implantation human embryo. *Mol. Hum. Reprod.*, **1**, 1571–1576.
- Castelbaum, A.J., Sawin, S.W., Bellardo, L.J. and Lessey, B.A. (1995) Endometrial integrin expression in women exposed to diethyl stilbestrol *in utero*. *Fertil. Steril.*, **63**, 1217–1221.
- Corless, C.L., Mendoza, A., Collins, T. and Lawler, J. (1992) Colocalization of thrombospondin and syndecan during murine development. *Dev. Dynamics*, **193**, 346–358.
- Fässler, R. and Meyer, M. (1995) Consequences of lack of β_1 integrin gene expression in mice. *Genes Dev.*, **9**, 1876–1908.
- Felding-Habermann, B. and Cheresch, D.A. (1993) Vitronectin and its receptors. *Curr. Opin. Cell Biol.*, **5**, 864–868.
- Fukuda, M.N., Sato, T., Nakayama, J. *et al.* (1995) Trophinin and tasin, a novel cell adhesion molecule complex with potential involvement in embryo implantation. *Genes Dev.*, **9**, 1199–1210.
- Glasser, S.R. and Mulholland, J. (1993) Receptivity is a polarity dependent special function of hormonally regulated uterine epithelial cells. *Microscopy Res. Technol.*, **25**, 106–120.
- Hey, N.A., Graham, R.A., Seif, M.W. and Aplin, J.D. (1994) The polymorphic epithelial mucin MUC1 in human endometrium is hormonally regulated with maximal expression in the implantation phase. *J. Clin. Endocrinol. Metab.*, **78**, 337–342.
- Hey, N.A., Li, T.-C., Devine, P.L., Graham, R.A. and Aplin, J.D. (1995) MUC1 in secretory phase endometrium: expression in precisely dated biopsies and flushings from normal and recurrent miscarriage patients. *Hum. Reprod.*, **10**, 2655–2662.
- Hynes, R.O. (1992) Integrins: versatility, modulation and signalling in cell adhesion. *Cell*, **69**, 11–25.
- Kimber, S.J., White, S., Cook, A. and Illingworth, I. (1993) The initiation of implantation: parallels between attachment of the embryo and neutrophil-endothelial interaction? In Mastroianni, L., Coelingh Bennink, H.J.T., Suzuki, S. and Verner, H.M. (eds), *Gamete and Embryo Quality*. Parthenon, London, pp. 171–198.
- Klentzeris, L.D., Bulmer, J.N., Tredosiewicz, L.K. *et al.* (1993) β_1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. *Hum. Reprod.*, **8**, 1223–1230.
- Lessey, B.A., Damjanovich, L., Coutifaris, C. *et al.* (1992) Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J. Clin. Invest.*, **90**, 188–195.
- Lessey, B.A., Castelbaum, A.J., Buck, C.A. *et al.* (1994) Further characterisation of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil. Steril.*, **62**, 497–506.
- Liaw, L., Skinner, M.P., Raines, E.W. *et al.* (1995) The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. *J. Clin. Invest.*, **95**, 713–724.
- Lindenberg, S., Sundberg, K., Kimber, S.J. and Lundblad, A. (1988) The milk oligosaccharide, lacto-N-fucopentaose I, inhibits attachment of mouse blastocysts on endometrial monolayers. *J. Reprod. Fertil.*, **83**, 149–158.
- Martin, L. and Finn, C. (1968) Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus. *J. Endocrinol.*, **41**, 363–371.
- McLean, J.W., Vestal, D.J., Cheresch, D.A. and Bodary, S.C. (1990) cDNA sequence of the human integrin β_5 subunit. *J. Biol. Chem.*, **265**, 17126–17131.
- Noyes, R.W., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1**, 3–25.
- O'Shea, K.S., Liu, L.-H.J., Kinnunen, L.H. and Dixit, V.M. (1990) Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo. *J. Cell Biol.*, **111**, 2713–2723.
- Ruck, P., Marzusch, K., Kaiserling, E. *et al.* (1994) Distribution of cell adhesion molecules in decidua of early human pregnancy. *Lab. Invest.*, **71**, 94–101.
- Shultz, J.F. and Armant, D.R. (1995) β_1 and β_3 -class integrins mediate fibronectin binding activity at the surface of developing mouse peri-implantation blastocysts. *J. Biol. Chem.*, **270**, 11522–11531.
- Stephens, L.E., Sutherland, A.E., Klimanskaya, I.V. *et al.* (1995) Deletion of β_1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev.*, **9**, 1883–1895.
- Sutherland, A.E., Calarco, P.G. and Damsky, C.H. (1993) Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development*, **119**, 1175–1186.
- Tabibzadeh, S. (1992) Patterns of expression of integrin molecules in human endometrium during the menstrual cycle. *Hum. Reprod.*, **7**, 876–882.
- Tabibzadeh, S. and Babaknia, A. (1992) The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Hum. Reprod.*, **10**, 1579–1602.
- Taskin, O., Brown, R.W., Young, D.C. *et al.* (1994) High doses of oral contraceptive do not alter endometrial β_1 and $\alpha_v\beta_3$ integrin in the late implantation window. *Fertil. Steril.*, **61**, 850–855.
- Turpeenniemi-Hujanen, T., Feinberg, R.F., Kaupilla, A. and Puistola, U. (1995) Extracellular matrix interactions in early human embryos: implications for normal implantation events. *Fertil. Steril.*, **64**, 132–138.
- Vićovac, Lj., Starkey, P.M. and Aplin, J.D. (1994) The effect of cytokines on prolactin production by human decidual stromal cells in culture. *J. Clin. Endocrinol. Metab.*, **79**, 1877–1882.
- Vogel, B.E., Lee, S.J., Hildebrand, A. *et al.* (1993) A novel integrin specificity exemplified by binding of the $\alpha_v\beta_5$ integrin to the basic domain of the HIV Tat protein and vitronectin. *J. Cell Biol.*, **121**, 461–468.
- Wickham, T.J., Mathial, P., Cheresch, D.A. and Nemerow, G.R. (1992) Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$: receptors for adenovirus internalisation. *Cell*, **73**, 309–319.
- Yelian, F.D., Yang, Y., Hirata, J.D. *et al.* (1995) Molecular interactions between fibronectin and integrins during mouse blastocyst outgrowth. *Mol. Reprod. Dev.*, **41**, 435–448.

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