ASSESSMENT OF DEVELOPMENT AND FUNCTIONAL CAPACITY OF TRANSPLANTED METANEPHROI

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List of abbreviations

ACE	Angiotensin converting enzyme
APC	Antigen presenting cell
ANOVA	Analysis of variance
APD	Ambulatory peritoneal dialysis
AQP	Aquaporin
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
AVP	Vasopressin
bFGF	basic Fibroblast growth factor
BSA	Bovine-serum albumin
BSU	Biological services unit
BW	Body weight
C_x	Clearance of substance x
CAPD	Continuous ambulatory peritoneal dialysis
CCD	Cortical collecting duct
CD31	Cluster of differentiation 31 (also called PECAM-1)
cDNA	complementary Deoxyribonucleic acid
CKF	Chronic kidney failure
Ct	Cycle time
DAB	Di-aminobenzedine
DBP	Diastolic blood pressure
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
dNTP	deoxynucleoside triphosphate
DTT	Dithiothreitol
DVR	Descending vasa recta
E_x	Embryonic day x
ECF	Extracellular fluid
ECFV	Extracellular fluid volume
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ENaC	Epithelial sodium channel
ERBF	Effective renal blood flow
ERPF	Effective renal plasma flow
ESRD	End-stage renal disease
FE	Fractional excretion
FGF	Fibroblast growth factor
Galα1,3Gal	Galactose-α1,3-galactose
GFR	Glomerular filtration rate
GM-CSF	Granular monocyte-colony stimulating factor
H&E	Haematoxylin & eosin
HD	Haemodialysis
HDAF	Human decay accelerating factor
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
IL-2	Interleukin-2
IMCD	Inner medullary collecting duct

IMS	Industrial methylated spirit
KO	Knockout
KW	Kidney weight
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant protein-1
mHC	Minor histocompatibility complex
mRNA	messenger Ribonucleic acid
MHC	Major histocompatibility complex
MOPS	3-N-Morpholinpropanesulfonicacid
MR	Mineralocorticoid receptor
NHE3	Sodium/hydrogen exchanger 3
NHP	Non-human primate
NKCC2	Sodium-potassium-2-chloride cotransporter type 2
OMCD	Outer-medullary collecting duct
PAH	Para-aminohippuric acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
PECAM-1	Platelet endothelial cell adhesion molecule-1
PFA	Paraformaldehyde
pmp	people per million population
PND	Postnatal day
P_x	Concentration of substance x in plasma
qPCR	Quantitative polymerase chain reaction
RBF	Renal blood flow
RECA-1	Rat endothelial cell antigen-1
RNA	Ribonucleic acid
RPF	Renal plasma flow
RRT	Renal replacement therapy
RT-PCR	Reverse transcriptase-polymerase chain reaction
RVR	Renal vascular resistance
SEM	Standard error of the mean
SPSS	Statistical package for the social sciences
TAL	Thick ascending limb
TBE	Tris-borate buffer
TDL	Thin descending limb
TEG	Tris-EGTA buffer
TGF-β	Transforming growth factor-β
Tx	Transplanted metanephros
URR	Urea reduction ratio
USRDS	United States Renal Data Service
UT-A	Urea transporter A
UV	Urine flow rate
U_xV	Excretion rate of substance x
UV/VIS	Ultra-violet/visible light
V_2	Vasopressin receptor
VEGF	Vascular endothelial growth factor
v α-SMA	Vacular α-smooth muscle actin

Abstract

Transplantation of embryonic kidneys (metanephroi) has been proposed as a potential solution to the problem of kidney donor shortage. The aim of this study was to characterise the physiological capacity of transplanted rat metanephroi and to determine the number and maturity of the tubules. Metanephroi from E15 Lewis rat embryos were transplanted adjacent to the abdominal aorta of uninephrectomised adult female Lewis rats. 21 days later, a single metanephros ureter was anastomosed to the host's urinary system. Three months later animals (n = 6) were prepared for standard clearance measurements. The right ureter and bladder were cannulated for collection of urine from the native kidney and transplanted metanephros, respectively. Transplanted metanephroi were explanted at 3 weeks or 4 months post-transplantation and processed for immunohistochemistry or PCR analysis. The clearance studies revealed that effective renal blood flow, standardised to kidney weight, was significantly lower in transplanted metanephroi compared with control adult kidneys (P<0.05) and that renal vascular resistance was significantly higher (P<0.05). Nephron number in transplanted metanephroi was significantly greater than that of E21 kidneys (P<0.05) but significantly less than PND1 kidneys (P<0.05). Immunohistochemistry identified localised aquaporin 1 and 2, ENaC and NKCC2 staining in transplanted metanephroi; positive staining for urea transporters A-1,2 and 3 was absent. Quantitative PCR showed that mRNA expression of these targets (excluding UT-A1 and 3) was not significantly different from E21 and PND1 expression levels (P>0.05). Finally, transplants were dosed with 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte colony stimulating factor (GM-CSF) in an attempt to promote arteriogenesis. Quantitative PCR showed that expression of vascular smooth muscle was not increased following dosing (P>0.05). Only UT-A1 and 2 showed a significantly higher expression following incubation in MCP-1 and GM-CSF (P<0.05). This study described the limited blood flow of transplanted metanephroi for the first time together with a tubular number and maturity similar to rat kidneys around time of birth.

Declaration

I confirm that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Mark. R. Dilworth

Copyright statement

Publications arising from this work

Marshall D, Dilworth MR, Clancy M, Bravery CA and Ashton N (2006). Increasing renal mass improves survival in anephric rats following metanephros transplantation. *Experimental Physiology*. In press DOI 10.1113 /expphysiol.2006.036319

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Dilworth MR, Clancy M, Marshall D and Ashton N (2006). Development of transplanted metanephroi: evaluation of glomerular number and expression of key transporters. 9th Annual Congress of the British Transplantation Society, Edinburgh, 2006: P2.

Dilworth MR, Clancy M, Marshall D, Bravery CA and Ashton N (2005). Transplanted metanephroi: evidence of renal function and expression of key transporters. *Journal of the American Society of Nephrology* **16:** 356A.

Dilworth MR, Clancy M, Marshall D, Bravery CA and Ashton N (2005). Renal function in transplanted metanephroi: A potential source of renal replacement therapy? Joint Renal Association / British Transplantation Society meeting, Belfast 2005, http://www.triangle3.org.uk/cgi-bin/absdb/absdb_view.cgi?AbstractID=RA5302

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" The great tragedy of Science- the slaying of a beautiful hypothesis by an ugly fact. "
Thomas H. Huxley, *English biologist (1825-1895)*

Chapter 1: General introduction

1.1 Background to the project

1.1.1 The need for organ donors

Chronic kidney failure (CKF) is defined as the progressive loss of renal function over months or years. CKF is thought to affect around 653 people per million population (pmp) in the UK (Ansell et al., 2004) with the situation in the United States even more serious at around 1403 pmp (Coresh et al., 2005; Meguid El Nahas and Bello, 2005). CKF has been subdivided into 5 stages, outlined in table 1.1.

Stage 1	Kidney damage (pathological abnormalities or abnormalities within blood and urine) with normal or raised GFR (≥ 90 mL per min per 1.73 m^2)
Stage 2	Kidney damage with GFR 60-89 mL per min per 1.73 m^2
Stage 3	GFR 30-59 mL per min per 1.73 m^2
Stage 4	GFR 15-29 mL per min per 1.73 m^2
Stage 5	GFR <15 mL per min per 1.73 m^2 (End stage renal failure)

Table 1.1. Stages of chronic kidney failure. As suggested by the US National Kidney Foundation and the Kidney Outcomes Quality Initiative. Adapted from El Nahas and Bello, 2005. GFR; glomerular filtration rate.

As can be seen in table 1.1, end stage renal disease is the 'end-point' of chronic kidney failure and is described as the loss of kidney function to around 10% or less of normal renal function. In the UK, around 100 pmp have been diagnosed with end stage renal disease (ESRD) (Ansell et al., 2004) of which the vast majority are over 65 years of age. This figure for

ESRD has doubled over the last 10 years and is expected to rise by between 5-8% annually. The situation in the United States is even more serious, with 336 pmp diagnosed with ESRD (USRDS, 2005). The major causes of ESRD are hypertension, type 2 diabetes and infections of the urinary tract, with the rise in ESRD reflecting the increase in type 2 diabetes worldwide. ESRD is fatal if untreated, so patients must undergo renal replacement therapy (RRT) in order to survive. At present, there are 2 major therapeutic strategies for RRT: either treatment by dialysis or transplantation of another kidney.

1.1.2 Dialysis treatment

Dialysis can be subdivided into 2 forms; peritoneal dialysis (PD) and haemodialysis (HD). Most patients can have either kind of dialysis and regularly switch between the 2 forms. Both forms rely on a membrane, which acts as a filter to replicate the kidney's actions in the regulation of salt and fluid balance and the excretion of metabolic waste products such as urea. Around 40% of patients on dialysis undergo PD whereby the process of dialysis actually occurs inside the body, using the peritoneum as the dialysate. Dialysis fluid is introduced into the peritoneal cavity and drained out after a few hours. There are 2 major forms of PD. In continuous ambulatory peritoneal dialysis (CAPD) the patient has dialysis fluid in their body 24 hours a day; the patient drains the fluid themselves 4 or 5 times a day. In another form, termed automated peritoneal dialysis (APD), a machine automatically changes the dialysis fluid. Both forms of

PD must be carried out daily. The major benefit of PD is that time spent in hospital is reduced considerably, although regular blood tests are required to monitor pH, urea and electrolyte concentrations as well as haematocrit and systemic blood pressure.

Haemodialysis involves the use of a dialysis machine. Blood is routed from a venous catheter and pumped into the dialysis machine, where the blood passes through the membrane and is returned back to the body. HD is usually performed around 3 times per week for 3-5 hours per session, usually under medical supervision at hospital. The current standards for haemodialysis, which state the limits for blood pressure and haemoglobin levels post-dialysis, are listed in table 1.2.

Standard	**Haemodialysis**
Dialysis adequacy	URR ≥ 65% or Kt/V ≥ 1.2
Haemoglobin	≥ 10g/dL in > 85% of patients
Systolic BP	≤ 160 mmHg (patients aged over 60) ≤ 140 mmHg (patients aged under 60)
Diastolic BP	≤ 90 mmHg

Table 1.2. Current standards for haemodialysis. URR = Urea reduction ratio which denotes the % fall in blood urea during a haemodialysis session. Kt/V is a marker for evaluation of renal function where K = renal urea clearance (mL/min), t = time per week (mins) and V = urea distribution volume (mL). Values for peritoneal dialysis are as for haemodialysis with the exception of dialysis adequacy, with Kt/V values of 1.7 for Continuous Ambulatory Peritoneal Dialysis (CAPD) and 2.0 for Automated Peritoneal Dialysis (APD). Taken, in part, from The UK Renal Registry fifth annual report (Ansell et al., 2002)

These guidelines are a relatively simple way of assessing the effectiveness of dialysis, since it is very difficult to compare dialysis with the complex physiology of a normal, healthy, functioning kidney. What is known is that haemodialysis can clear urea from the blood at a rate of between 100 and 225 ml/min (Guyton and Hall, 2000). This compares very favourably with 2 normal functioning kidneys which together can clear urea at around 70 ml/min. However, this clearance capacity should be considered in the context of 3 dialysis sessions of 3-5 hours per week. Similarly, it has been estimated that dialysis is equivalent to a glomerular filtration rate (GFR) of around 10 mL/min/1.73m^2, around 10-15% of the GFR of a normal, functioning adult human kidney (Kuhlmann et al., 2001). With this in mind, it becomes clear that the artificial kidney, whilst sustaining life, falls some way short in terms of replacing the normal kidney. Although dialysis is able to filter the blood sufficiently to prolong life, it is unable to perform many of the multiple actions of the kidney. These include the production of vitamin D and erythropoietin, for the regulation of erythrocyte production. As such, patients on dialysis have to be closely monitored for changes in haematocrit and signs of anaemia, and they are supplemented with erythropoietin as a result.

Treatment by dialysis also has numerous side effects, such as risk of hernias, bacterial infection and peritonitis in PD, nausea and lethargy, muscle cramps and risk of clotting in HD. One of the main problems in HD is vascular access, since new sites are required at regular intervals. In both forms of dialysis, the patient has a relatively 'poor' quality of life since they require frequent medical supervision, meaning they often cannot

stray far from home. As such, renal transplantation is a more 'attractive' form of RRT, hence the ever increasing requirement for healthy donor kidneys.

1.1.3 Transplantation and the organ shortage

Latest statistics show that, in the UK alone, over 1300 kidney transplants were performed in 2005 (uktransplant.org.uk). However, this is a relatively small number by comparison with a waiting list of over 5700 patients. Thus, 3 out of 4 patients on the waiting list are unlikely to receive a renal transplant. Clearly, at the current rate of new ESRD cases, this waiting list is only likely to increase further.

Transplantation of kidneys is often the preferential form of RRT, given that the one-year survival rate for patients with renal transplants is 97%, compared to 84% for dialysis patients (Wolfe et al., 1999; Ansell et al., 2004). Furthermore, the one-year survival rate of patients who have undergone pre-emptive renal transplants (100%) has been shown to be greater than that of patients who had undergone dialysis prior to transplantation (89% survival) (Grochowiecki et al., 2006). Hence the ideal RRT would appear to be transplantation of a donor kidney as soon as possible.

Despite concerted efforts to increase the number of donor organs available for transplantation, from either living or cadaveric donors, there still remains a massive deficit. This is referred to as the organ shortage. Clearly, whilst this deficit remains, there is a requirement for alternative

transplantation strategies to be investigated. Prior to discussing these however, it is first important to discuss the types of immunological rejection that have to be avoided in order for a transplant to function.

1.1.4 Immunological rejection of transplants

1.1.4.1 Hyperacute rejection

Hyperacute rejection is the rapid destruction of the graft, usually commencing after reperfusion of the tissue. It is characterised by the formation of platelet thrombi (Platt and Holzknecht, 1994; Samstein and Platt, 2001). In the case of the kidney, this often occurs within the glomerular capillaries and small blood vessels. Hyperacute rejection is particularly common in xenotransplantation, the transplantation of an organ across the species barrier, and is initiated by the binding of xenoreactive natural antibodies. These antibodies are present within the recipient's circulation despite the recipient not having been exposed previously to the xeno-antigen (Boyden, 1966). In the case of porcine transplantation, most of these xenoreactive natural antibodies are directed against galactose-α-1,3-galactose (Galα1,3Gal), a saccharide expressed on the vascular endothelium of most mammals, with the exception of humans, apes and old world monkeys (Galili et al., 1985; Galili et al., 1988). It is known that antibodies directed against Galα1,3Gal comprise up to 95% of the total number of xenoreactive antibodies and that hyperacute rejection, in pig to baboon transplants, can be prevented by the elimination

of antibodies directed against Galα1,3Gal (Lin et al., 1998; Lin et al., 2000).

It is known that binding of xenoreactive natural antibodies activates the complement system, ultimately leading to the disruption of the endothelium and activation and adhesion of platelets. It is thought that this occurs largely due to an inability of porcine complement regulatory proteins to regulate/inhibit human or primate complement (Platt et al., 1990) meaning that complement activation within the recipient is largely unchecked and hyperacute rejection ensues.

Hyperacute rejection can be prevented by a reduction in the number of xenoreactive natural antibodies, mainly by perfusion of the host's blood through a Galα1,3Gal adsorption column prior to xenotransplantation (Sablinski et al., 1997; Lin et al., 1998; Lin et al., 2000). Hyperacute rejection can also be prevented by the inhibition of the complement cascade (Weisman et al., 1990; Pruitt et al., 1994) or the use of transgenic pigs expressing compatible complement regulatory proteins such as human decay accelerating factor (HDAF), as described later (Zaidi et al., 1998a).

1.1.4.2 Acute vascular rejection

Acute vascular rejection is a different type of immune response, which occurs over a period of days or months. It is usually caused by either a mismatch between donor and recipient in the human leukocyte antigen (HLA) on the cell surface (Opelz et al., 1973) or, in the case of a

xenotransplant, due to the presence of xenoreactive antibodies (Cotterell et al., 1995).

Acute vascular rejection is characterised by intravascular thrombosis (due to the presence of fibrin), focal ischaemia and necrosis and swelling of the endothelial cells (Porter, 1967; McCurry et al., 1997). Some cases of acute vascular rejection may show the presence of monocytes (Blakely et al., 1994) and natural killer cells (Malyguine et al., 1996). In terms of the transplantation of xenografts, it is thought that xenoreactive antibodies within the recipient activate donor endothelial cells to cause rejection (Hattori et al., 1989; Cotterell et al., 1995), possibly by the actions of macrophages and natural killer cells (Blakely et al., 1994; Malyguine et al., 1996).

In allotransplantation, the transplantation of tissue from one individual to another of the same species, acute vascular rejection can be avoided or delayed by finding the closest possible HLA match between recipient and donor, often a relative, and by the use of immunosuppressive drugs. In xenotransplantation, similar to hyperacute rejection, xenoreactive antibodies must again be depleted to avoid acute vascular rejection. This has been achieved by the use of immunoadsorbant columns, splenectomy and immunosuppression (Leventhal et al., 1995; Taniguchi et al., 1996; Samstein and Platt, 2001). Success in preventing hyperacute and acute vascular rejection has been boosted significantly by the generation of pigs in which the Galα1,3Gal gene is either disrupted (Dai et al., 2002) or completely knocked-out (Lai et al., 2002; Phelps et al., 2003). Although these studies showed that the pigs and their organs appeared to be

normal and healthy, further research is required to identify whether knocking-out the Galα1,3Gal gene causes any side-effects not yet reported.

1.1.4.3 Chronic (cellular) rejection

Assuming the accommodation of a transplanted organ has been reached (avoidance of hyperacute and acute vascular rejection), most grafts are eventually rejected by the process of chronic or cellular rejection over months or years. Cellular rejection is the most common form of rejection in allografts and it is thought that the chronic rejection of transplants may be due to the minor histocompatibility complex antigens (mHCs) such as the H-Y gene on the male chromosome (Koulack et al., 1996). There is a strong cellular response by the T lymphocytes (T cells) and macrophages (Waltzer et al., 1987; Alpers et al., 1990) of the recipient's immune system against both allografts and xenografts, which ultimately leads to the destruction of the graft.

In both xenografts and allografts, the T lymphocytes of the recipient recognise the major histocompatibility complex (MHC) antigens of the donor and bind to them directly (Murray et al., 1994; Yamada et al., 1995), causing the recruitment of macrophages and natural killer cells and 'rejection' of the graft (MacPherson and Christmas, 1984; Malyguine et al., 1996).

Chronic rejection of the transplant is largely avoided, at present, by the administration of immunosuppressive drugs such as Rapamycin (also known as Sirolimus) which inhibits the production of interleukin-2 (IL-2)

and thus the activation of T and B lymphocytes (Dumont et al., 1990; Gonzalez et al., 2001). Another major research area dedicated to preventing cellular rejection of graft examines the idea of immunological 'tolerance', where the immune system of the recipient fails to attack an antigen from the graft. Methods of achieving immunological tolerance range from T cell depletion (Contreras et al., 1998) to blockade of some of the receptors required for T cell activation by B lymphocytes (Kirk et al., 1999).

Although prevention of chronic rejection is still being extensively researched, a solution to this problem remains elusive, meaning that most grafts will eventually succumb to cellular rejection. This can be delayed by the use of immunosuppressant drugs but ultimately, other transplantation strategies will likely be required.

1.2 Alternative transplantation strategies

1.2.1 Xenotransplantation

Xenotransplantation has been proposed as a potential solution to the problem of donor organ shortage. In the previous section some of the problems associated with host-graft rejection of xenotransplanted organs were outlined. This section discusses the advantages of using xeno-transplants and also some of the physiological problems that are likely to be encountered.

It was thought initially that xenotransplantation would have numerous advantages. These included the fact that patients who had previously been denied a transplant, such as the elderly and those with underlying medical problems, would finally have access to a donor organ. Another positive advantage of this approach would be that the actual transplant procedure could be planned in advance, as it would be possible to harvest the donor organ on demand.

Initially, it was assumed that if xenotransplantation was to be successful, the donor species would have to be a non-human primate (NHP), given their phylogenetic proximity to humans. It had been shown in the 1960's that xenografts of NHP kidneys into man could survive for months, even with the limited immunosuppression available at that time (Reemtsma et al., 1964). However, there are problems with the choice of NHPs as the source of donor organs. Many primate species are now critically endangered, meaning that the supply of organs would not be sufficient to meet demand. Also, there is a risk of infection such as simian immunodeficiency virus/ human immunodeficiency virus and the herpes B virus (Huff and Barry, 2003) when transplanting a NHP kidney into a human.

As a result of these concerns, the suitability of other species as potential kidney donors has been investigated. Pigs are one species that have received considerable attention. Pigs are plentiful and share a number of anatomical and physiological similarities with humans. For example, pig kidneys are of a similar size and have a similar structure to human kidneys (Kirkman, 1989). Glomerular filtration rate (up to 175 ml/min) and maximal

concentrating ability (1080 mosmol/L) are also close to the values for human kidneys (Kirkman, 1989). Pigs also have the significant advantage of being able to be bred 'pathogen-free' (Betts, 1962). Thus, the risk of infection, particularly in immunosuppressed patients, is much reduced. Pigs can also be genetically manipulated to express extrinsic genes. For example, pigs have been bred to express human decay-accelerating factor (HDAF) in order to prevent hyperacute rejection following kidney xenotransplantation, by the inactivation of the recipient's complex cascade (Schmoeckel et al., 1996). The subsequent kidney HDAF-xenograft survived for over 10 weeks in nephrectomised cynomolgus monkeys and showed demonstrable function (Zaidi et al., 1998a). According to these data, the use of porcine kidney xenografts is promising in the future. However, there are numerous physiological problems to be overcome before this transplantation strategy becomes a reality.

One of the first hurdles to be overcome is the problem that hormones or protein cascades produced by the xenograft may not interact fully with the recipient. An example of this is the action of renin. It has been shown that renin produced by the human recipient is much more effective at causing the conversion of angiotensinogen to angiotensin I than heterologous porcine renin (Sen et al., 1971). This could cause long-term problems for the control of arterial pressure within the recipient. There is also evidence that porcine erythropoietin is unable to stimulate human erythrocyte production (Zaidi et al., 1998b) and thus human erythropoietin may need to be administered to maintain red blood cell production. A final major concern is that porcine thrombomodulin, a key integral membrane protein

in the anti-coagulant pathway, has been shown to fail to interact fully with human protein C. This could result in severe dysfunction of the anti-coagulant pathway and an increased risk of coagulation in the recipient (Lawson et al., 1997). Given the fact that transgenic pigs can now be produced, it is possible that these hurdles can all be cleared. However, these physiological problems, together with the immunological avoidance of rejection, mean there is scope for other transplantation strategies to be examined. One such strategy is the transplantation of embryonic tissue, and in this example, embryonic kidneys (metanephroi).

1.2.2 The transplantation of embryonic kidneys

1.2.2.1 Development of the kidney

Prior to discussing the merits of transplanting metanephroi, it is important to briefly discuss how the kidney develops and the timescale involved.

In mammals, the metanephros is the last of the three renal excretory organs to develop, after the pronephros and mesonephros. It is the metanephros that goes on to form the adult kidney. The metanephros originates in the fifth week of gestation in humans, around embryonic day 12 (E12) in the rat and between E24-E28 in the pig. This compares with a gestation period of about 22 days in the rat and 116 days in the pig.

Development of the nephrons within the metanephros begins when the ureteric bud, an outgrowth of the Wolffian (mesonephric) duct, stimulates a group of mesenchymal cells (Saxen, 1987; Ekblom, 1992). The mesenchymal cells (metanephric mesenchyme) begin to proliferate and

differentiate into epithelial cells, forming vesicles. Cells within these vesicles change their shape, which results in the formation of a cleft, termed the comma-shaped body. When another cleft appears at the opposite end of the vesicle, the structure is termed an s-shaped body. Finally the s-shaped body begins to elongate. Within this s-shaped body structure are the cell types that will eventually form the glomerulus, proximal tubule, loop structures and distal tubule. The distal ends of the ureteric bud form the collecting system of the nephron and it is the fusing of the ureteric bud and the s-shaped body that signifies the formation of the complete, but immature, nephron. The ureter and pelvis of the kidney are derived from the proximal end of the ureteric bud (Saxen, 1987). This process of nephrogenesis is known to be complete at birth in humans (Hinchliffe et al., 1991) whereas, in rats, nephrogenesis continues until around 10-11 days after birth (Kavlock and Gray, 1982). In pigs, nephrogenesis is not complete until around 3-4 weeks after birth (Friis, 1980).

As the rat is the species of choice in the current project, the time-line of the development of the rat metanephros is discussed here. The metanephros first appears at around E12 in the rat. Mesenchyme can be identified first at E14, with comma-shaped bodies starting to appear between E15-E16. S-shaped bodies are first observed between E16 and E17, with glomeruli first distinguishable at E17-E18, around the same time as the first identification of any blood vessels. Identifiable tubules are not present until E17 (Bertram et al., 2000).

Figure 1.1 shows H&E sections of an E15 rat metanephros and a postnatal day 1 rat kidney in order to highlight the rapid development that occurs between these time-points (8 days).

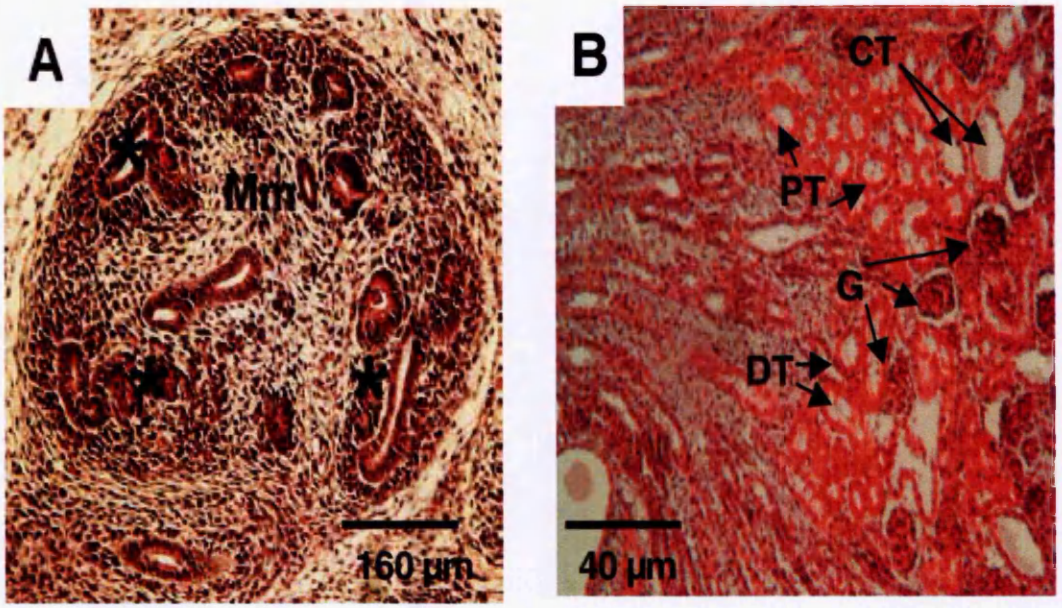

Figure 1.1. H&E images comparing E15 and PND1 kidneys. Haematoxylin and eosin (H&E) sections of a rat embryonic day 15 (E15) metanephros (A) and a rat postnatal day 1 (PND1) kidney (B). Undifferentiated mesenteric mesenchyme (Mm) and rudimentary epithelial structures (asterisks) can be seen in (A) whilst glomeruli (G), proximal convoluted tubules (PT), distal convoluted tubules (DT) and collecting tubules (CT) can all be observed in PND1 kidney (B). Original magnifications were x25 (A) and x100 (B). Images were produced in collaboration with Intercytex Ltd.

1.2.2.2 Rationale for transplanting metanephroi

One RRT strategy that is being investigated currently is the transplantation of embryonic kidneys (metanephroi), at a stage before the metanephros becomes vascularised. There are three major reasons why it was thought that transplanting embryonic kidneys, rather than adult renal tissue, would

be beneficial to the recipient. Firstly, the antigen presenting cells (APCs), such as macrophages, that are involved in graft-host rejection are not thought to be present in early embryonic tissue (Naito, 1993). Secondly, it has been suggested that the antigens present on the donor, such as the MHC molecules, may not be expressed as highly in embryonic tissue compared to adult expression levels (Statter et al., 1989). Finally, it is thought that the blood supplying the metanephros is derived largely from the host, via angiogenesis, rather than from the transplant itself, via vasculogenesis (Rogers and Hammerman, 2001b). This latter point has been the subject of much debate, with others suggesting that vasculogenesis plays a role in the vascularisation of metanephric transplants (Abrahamson et al., 1998). However, a recent paper showed that following transplantation of pig metanephroi into rats, only rat endothelial cells were present in the porcine tissue (Takeda et al., 2006). This strongly suggests that the majority, if not all, of the vasculature supplying the transplanted metanephros was of host origin. This means that the metanephros is, in essence, a chimeric organ. If this is indeed the case, the risks of hyperacute and acute vascular rejection would be diminished considerably.

Clearly, the 3 points listed make the transplantation of metanephroi an attractive proposition. However, in order for this strategy to achieve a level of life-prolonging function, the transplanted metanephroi have to be able to avoid graft-host rejection (either with or without immunosuppression), grow and develop into a structurally sound and recognisable kidney and show physiological function, at the very least, comparable with dialysis.

1.2.2.3 Growth of transplanted metanephroi and avoidance of immunological rejection

Research into the transplantation of metanephroi has been pioneered by the lab of Dr Marc Hammerman in the United States. His group and others have shown that, following transplantation into either rats or mice and unilateral nephrectomy of the host animal, metanephroi from rats, mice, pigs and humans all grow into kidney-like structures with identifiable glomeruli and tubular structures (Rogers et al., 1998; Rogers and Hammerman, 2001a; Rogers et al., 2001; Dekel et al., 2002; Dekel et al., 2003; Armstrong et al., 2005; Marshall et al., 2005). Some images of transplanted metanephroi showing these structures can be seen in Figure 1.2.

The successful growth and development of transplanted metanephroi has only been achieved as a result of numerous refinements to the transplantation procedure. Considerations included the embryonic time point at which metanephroi were transplanted, whether growth factors were added prior to transplantation, the site of transplantation and the species of both donor and recipient.

A number of transplantation sites have been employed, including the anterior eye chamber (Abrahamson et al., 1998), the omentum (Rogers et al., 1998; Dekel et al., 2003; Rogers et al., 2003) and the renal capsule (Foglia et al., 1986; Woolf et al., 1990). In the study by Woolf *et al* (1990) slices of metanephroi, rather than a whole metanephros, were transplanted into the cortex of existing adult kidneys through fine incisions.

In this study, identifiable kidney tubular structures were observed in the metanephric 'slices', although the tubules of the donor metanephroi failed to connect with the tubules of the adult kidney of the recipient.

In terms of the optimal age at which embryonic kidneys should be transplanted, it was apparent that rejection of metanephroi was avoided if tissue was transplanted prior to vascularisation. The most successful developmental stage was embryonic day 15 for rats (Rogers et al., 1998; Rogers and Hammerman, 2001a; Marshall et al., 2005), E12.5-13 for mice (Woolf et al., 1990; Armstrong et al., 2005), E27-28 for pigs and 7-8 weeks of gestational age for humans (Dekel et al., 2003). Transplantation of metanephroi at later embryonic ages generally led to immunological rejection and necrosis in immunocompetent recipients.

Though growth of the metanephroi occured in all of the above studies, it appears that growth was stunted, with transplants still only around 10% of the size of adult kidneys a few months after transplantation (Rogers et al., 1998; Marshall et al., 2005). The implications of this growth retardation will be discussed in much greater detail in later chapters.

In order to attempt to increase growth of transplants, metanephroi were incubated with a 12-growth factor cocktail prior to transplantation (Rogers and Hammerman, 2001a; Rogers and Hammerman, 2001b). Included in this growth factor cocktail were; vascular endothelial growth factor (VEGF), insulin-like growth factor I and II, hepatocyte growth factor and transforming growth factor α. Blocking these factors inhibits growth of metanephroi *in vitro* (Rogers et al., 1999; Hammerman, 2000) whilst blocking VEGF *in vivo* inhibits renal vascularisation (Kitamoto et al., 1997).

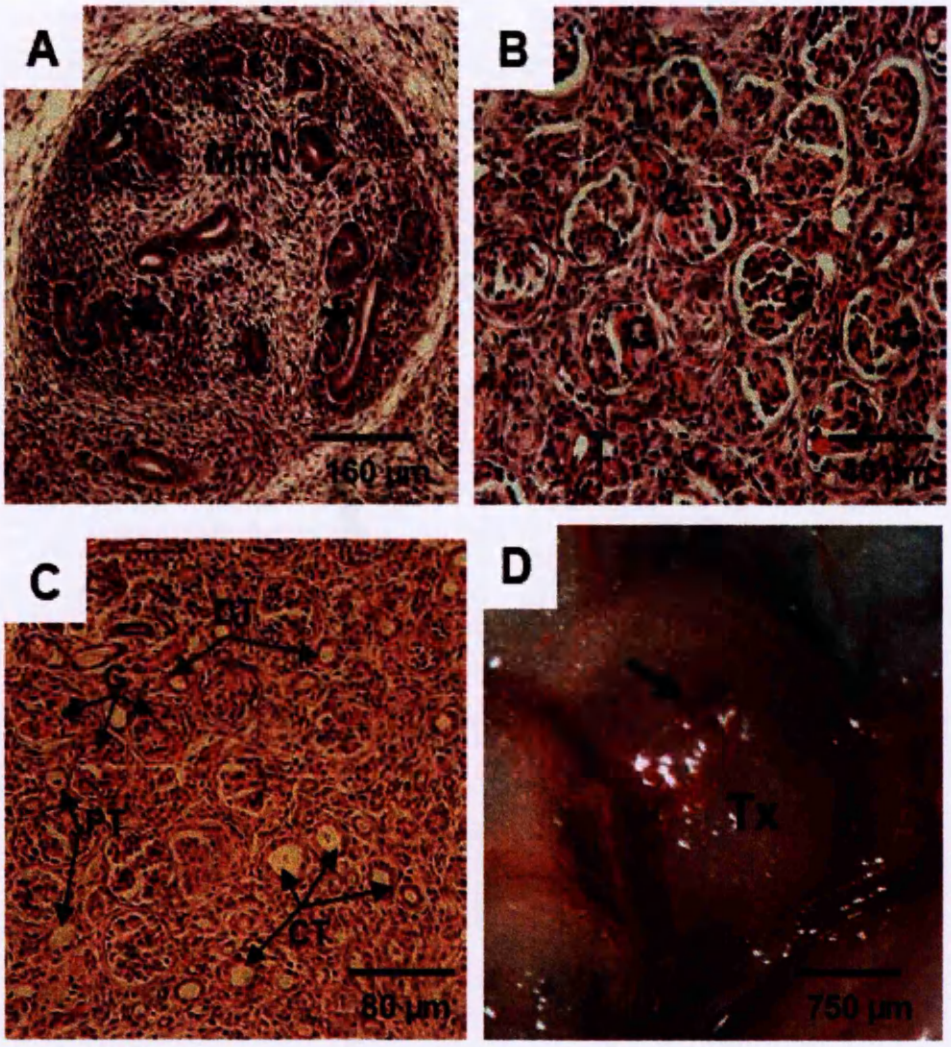

Figure 1.2. Images of E15 kidney and transplanted metanephroi. Haematoxylin and eosin (H&E) sections of a rat embryonic day 15 (E15) metanephros (A) showing undifferentiated metanephric mesenchyme (Mm) and rudimentary epithelial structures (asterisks), and H&E sections of a transplanted metanephros 21 days post-transplantation (B-C). Visible are glomeruli (G), proximal convoluted tubules (PT), distal convoluted tubules (DT) and collecting tubules (CT). A photograph of a transplanted metanephros (Tx) 21 days post-transplantation shows a blood vessel originating from the host blood vessel (arrow) (D). Original magnification for (A) was x25, (B) x100 and (C) x50. Images courtesy of Intercytex ltd.

Although transplanted metanephroi are able to grow if they are not incubated in the growth factor cocktail prior to transplantation (Rogers et al., 1998; Rogers et al., 2001), incubation in this mixture of growth factors does enhance growth of the metanephroi (Rogers and Hammerman, 2001a; Rogers and Hammerman, 2001b; Marshall et al., 2005).

It is apparent from the work of Hammerman and colleagues that transplanted metanephroi can be stimulated to grow in the host. This growth appears to improve following removal of a substantial amount of native renal mass (Rogers et al., 1998) and by transplanting metanephroi into a 'growth factor' rich environment such as a pregnant animal (Armstrong et al., 2005). What is less clear, however, is how long do transplanted metanephroi continue to grow following transplantation, assuming rejection has been avoided? Unpublished work from this laboratory suggests that transplanted rat metanephroi stop growing somewhere between 3 weeks and 6 weeks post-transplantation. Certainly, by 3 months post-transplant, the growth of metanephroi has ceased (M.Clancy, M.Dilworth, D.Marshall and N.Ashton, unpublished observations).

Another consideration in the development of a successful transplantation protocol was how to avoid immunological rejection. One strategy involves the use of a syngeneic animal model, in which a highly inbred strain of rat is used, such as the Lewis rat (Marshall et al., 2005). In this model, immunosuppression of the host is not required. This strategy has also been shown to be effective in a murine transplant model (Armstrong et al., 2005). Furthermore it has been reported that metanephroi from an outbred

strain of rat (Sprague-Dawley) can be transplanted successfully into a rat of the same strain without the need for immunosuppressive drugs (Rogers et al., 1998). The metanephroi grew and differentiated into recognisable kidney-like structures. In contrast, fully developed kidneys transplanted from one Sprague-Dawley rat to another underwent acute vascular rejection within 7 days (Rogers et al., 1998). This group has also shown that rat metanephroi are able to avoid rejection when transplanted across the major histocompatibility complex (MHC), again without immunosuppression, due to a state of immune-tolerance (Rogers et al., 2001). This was shown by using rats as donor and recipient that expressed different class I and II MHC antigens. It was thought that due to a lack of antigen presenting cells on the metanephroi, T cells from the host largely 'ignored' the transplant and rejection was not forthcoming.

Most of these studies involved the transplantation of metanephroi into a host of the same species. If this approach is to be of clinical value it is important to know if metanephroi can be transplanted successfully from one species to another. At the present time, some form of immunosuppression or immunological tolerance is required in order to avoid rejection of xenotransplanted metanephroi. This has been shown following transplantation of human and porcine metanephroi into immunodeficient mice (Dekel et al., 2003). In this study, transplants grew and developed into a kidney-like structure when transplanted into immunodeficient mice, but underwent acute rejection within 2 weeks of transplantation into immunocompetent mice. It has also been shown that porcine metanephroi were able to grow and differentiate following

transplantation into rats, but only with the administration of tolerance-inducing agents such as anti-CD45, and immunosuppressive drugs such as tacrolimus (Takeda et al., 2006). Similarly, rat metanephroi were able to avoid rejection following transplantation into mice, but only when tolerance-inducing agents, that immunocompromise the host in some way, were administered (Rogers and Hammerman, 2001b).

These studies have shown that the degree of antigenic presentation by developing kidneys is much less than that observed in fully developed kidneys. This reduced antigenicity means that metanephroi can be transplanted between outbred strains of the same species without the need for immunosuppression. However, at the present time, xenotransplanted metanephroi are only able to survive as long as the host is immunocompromised sufficiently.

1.2.2.4 Physiology of transplanted metanephroi

Many of the studies described so far have focused on the avoidance of rejection of the transplanted metanephroi. Since the main focus of the current study is the physiology of transplanted metanephroi, it is important to consider the limited physiological measurements that have been carried out to date. Following transplantation and growth of the metanephros, it is possible to 'connect' the free-end of the ureter from the transplant to the free-end of the ureter from a host kidney that has been removed previously. This is known as a ureteroureterostomy. This procedure allows any urine produced by the metanephros to flow into the bladder from which it may be collected (a cannula inserted into the ureter of the one

remaining native kidney diverts this urine away from the bladder). Using this approach, glomerular filtration rate (GFR) and urine flow rate (UV) have been determined. Most of these studies have been carried out in a rat-rat model of transplantation, without the need for immunosuppressive strategies.

The first measurement of glomerular filtration rate in transplanted rat metanephroi was reported as 2.4 µl min^{-1} g kid wt^{-1} (Rogers et al., 1998). This figure increased to 30.1 µl min^{-1} g kid wt^{-1} in animals that had undergone a unilateral nephrectomy together with partial renal infarction of the one remaining native kidney. These figures were between 0.3 and 4% of the GFR of an adult rat control kidney (750 µl min^{-1} g kid wt^{-1}). Mean urine flow rate (UV) in transplanted metanephroi was around 3 µl min^{-1} g kid wt^{-1}.

Exposure of the metanephros to the 12 growth-factor cocktail described above further enhanced GFR. Filtration by untreated metanephroi was 3.8- 4.3 µl min^{-1} g kid wt^{-1} whereas that of metanephroi incubated in the growth-factor cocktail prior to transplantation was 11-12 µl min^{-1} g kid wt^{-1} (Rogers and Hammerman, 2001a). Considering that these measurements were made in a rat with unilateral nephrectomy (rather than subtotal nephrectomy) GFR showed an improvement compared with the previous experiment (2.4 µl min^{-1} g kid wt^{-1}). The reason for this is unknown, but may simply be due to greater expertise in the transplantation protocol as a result of more experience with the technique. In this experiment, it was also shown that metanephroi could be preserved *in vitro* for up to 3 days prior to transplantation with no alteration in the subsequent GFR (4-11 µl

min^{-1} g kid wt^{-1} compared with 3.8-12 µl min^{-1} g kid wt^{-1} following preservation *in vitro* for 3 days). Clearly, it will be of benefit if metanephroi can be stored prior to transplantation, enabling them to be transported over some distance before implantation.

The GFR values described above were obtained in anaesthetised rats. In conscious rats, GFR was improved again, at around 35 µl min^{-1} g kid wt^{-1}, approximately 9% of the GFR value of a normal rat kidney, whilst UV was between 10-23 µl min^{-1} g kid wt^{-1} (Rogers et al., 2001).

Another series of experiments showed that the site of transplantation was also important in determining the subsequent degree of physiological function displayed by the metanephros (Marshall et al., 2005). In this study, it was observed that metanephroi transplanted adjacent to the aorta had the highest GFRs, equivalent to 11% of the GFR of adult kidneys. This is compared with GFRs of only 0.2% of normal at the omentum, 1.7% adjacent to the iliac vessels and 3.1% adjacent to the renal vessels.

Clearly, these GFR values are not of sufficient magnitude to sustain life without other RRT such as dialysis. Nonetheless, it is worth noting that dialysis is only equivalent to a GFR of around 10-15% of a functioning adult kidney (Kuhlmann et al., 2001). Furthermore, multiple transplants could theoretically undergo ureteroureterostomy to provide cumulative function. So, this technology may be closer to life-sustaining function than it first appears.

Apart from GFR and UV, the only other physiological parameter which has been reported is the concentration of urea. This has been measured in the host's plasma, urine produced by the metanephros and cyst fluid, which is

fluid collected from a dilated ureter that has not been connected and resembles a cyst, from rat, porcine and human metanephroi. Urea concentrations in these studies can be seen in Table 1.3.

	Urea concentration (mg/dl)		
Reference	**Plasma**	**Cyst fluid**	**Metanephric urine**
Rogers *et al*, 1998	53.8 ± 6.3	136 ± 16	800 ± 72
Dekel *et al*, 2003	45 ± 8	518 ± 169	N/A

Table 1.3. Previously measured urea concentrations. Assessment of mean urea concentration within plasma (n = 7), cyst fluid (dilated non-connected ureter, n = 7), and urine produced by the metanephros (metanephric urine, n = 7) in rat metanephroi (Rogers et al., 1998) and mean urea concentration in plasma (n = 6) and cyst fluid (n = 6) from human and porcine metanephroi (Dekel et al., 2003). All values are expressed as mean ± SEM. The rat was the host species in both studies.

These studies showed, for the first time, that some degree of urine (urea) concentration was occurring within transplanted metanephroi. The mechanisms involved, beit facilitated diffusion via urea transporters or via the movement of water, were not explored in these studies and require further investigation.

Although considerable progress has been made towards developing metanephric transplantation as a RRT, there are large 'gaps' in our knowledge. In particular, little is known about the physiology and functional capacity of transplanted metanephroi. For example, renal blood flow, vascular resistance and electrolyte excretion rates have yet to be reported.

Also, although it is known that transplanted embryonic kidneys show stunted growth, it is not clear if the number or maturity of tubules is also diminished. Such information would be extremely useful in determining if there is scope for improving functional capacity or increasing tubular function in transplanting metanephroi. This information will also provide a valuable baseline for comparison with the efficacy of growth enhancing strategies.

1.3 Aims of the project

The overall aim of this study was to take the next logical step in the development of this transplant model and characterise the physiological capacity of transplanted rat metanephroi. This included a determination of the degree of maturity of the nephrons. The first aim of the work was to gather a set of baseline physiological measurements for transplanted rat metanephroi. These included measurements of haemodynamics, GFR, renal vascular resistance, urine flow and electrolyte excretion rates.

The second aim was to assess the nephrogenic state and maturity of the tubules. This was achieved by counting glomerular, and thus nephron, number within the transplants and by examining the protein and mRNA expression of some of the transporters and channels crucial to the concentration of urine. These included the urea transporters and the aquaporin water channels. All of these results were compared with data from E21 and PND1 rat kidneys in order to establish the stage of development.

The final aim was to improve the blood supply to the transplants by encouraging growth of larger arteries from smaller arterioles (arteriogenesis).

All of these experiments were carried out using a syngeneic small animal model (Lewis rat-Lewis rat), in order to avoid the risk of immunological rejection of the metanephroi by the recipient. Female rats were chosen as hosts given the increased circulating levels of hormones such as oestrogen and progesterone in this sex. It was hypothesised that these increased concentrations of hormones would improve the growth of transplanted metanephroi.

Chapter 2: Function of transplanted metanephroi in vivo

2.1 Introduction

2.1.1 Previous research on transplanted metanephroi in vivo

Rat metanephroi have been shown to grow, undergo nephrogenesis and develop into vascularised kidneys with identifiable glomerular and tubular structures following transplantation into rats of the same or different strains (Rogers et al., 1998; Rogers and Hammerman, 2001a; Rogers et al., 2001; Marshall et al., 2005) or into mice (Rogers and Hammerman, 2001b). These early studies tended to focus on preventing rejection of the transplant rather than assessing the physiological performance of the metanephroi. As a result, metanephroi have been successfully transplanted across the major histocompatibility complex in rats, whilst avoiding rejection (Rogers et al., 2001).

Other published literature has taken this technology even further, with metanephroi being transplanted across the species barrier (Rogers and Hammerman, 2001b; Dekel et al., 2003). In both of these papers, the mouse was the host species of choice and was either immunocompromised or dosed with tolerance-inducing agents. This highlights the difficulty in sustaining renal xeno-transplants, even when transplanting at a stage prior to vascularisation of the transplant. The work by Dekel, Burakova *et al* (2003) involved the transplantation of human and porcine metanephroi into immuno-compromised mice, a significant step forward. Dekel showed that human and porcine metanephroi, following transplantation, developed into kidney-like structures with minimal necrosis.

Despite the main focus of all of these studies being on the ability to avoid rejection of the transplanted metanephroi, there are some published functional data, albeit limited, from rat-rat transplantation studies. In these reports (Rogers et al., 1998; Rogers and Hammerman, 2001a; Rogers et al., 2001) glomerular filtration rate (GFR), urine flow rate (UV) and urea concentrations were all measured. GFR measurements ranged from 2 to 35 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$. Notably, GFRs in the transplanted metanephroi were greater in animals that had undergone a partial nephrectomy of the one remaining native kidney in addition to a unilateral nephrectomy (30.1 ± 8.7 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$ compared to 2.42 ± 8.7 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$) (Rogers et al., 1998). Urine flow rates ranged from 2 μl to 11.5 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$. Concentrations of urea within plasma, cyst fluid (the fluid-filled dilated ureter present at around 3 weeks post-transplant) and urine produced by rat, human and porcine metanephroi have also been measured previously (Rogers et al., 1998; Dekel et al., 2003). The ranges of these values were as follows; serum, 45-58 mg/dL and cyst fluid, 136-518 mg/dL. The urea concentration of metanephric urine has been measured in only one previous study, with a value of 800 ± 72 mg/dL (Rogers et al., 1998).

To date, there is no published information on blood flow through the transplanted metanephroi and thus there has been no estimation of renal vascular resistance (RVR). RVR is a useful physiological measurement in this context, as it provides further information about blood flow through the metanephros. Typically, RVR is elevated as a result of vasoconstriction of the renal vasculature, or through obstruction by an atheromatous plaque. In the transplanted metanephros, it may also be indicative of the degree of

vascularisation and calibre of the blood vessel(s) supplying the metanephros. Similarly, there have been no published reports of osmolar and electrolyte excretion rates by transplanted metanephroi. Accordingly, the aim of this study was to further quantify the functional capacity of transplanted metanephroi. Before this could be achieved, it was necessary to gather baseline renal function measurements for Lewis rats as this strain has not been used widely in renal function studies, and to establish a reliable, and repeatable clearance protocol, hence a pilot study was carried out.

2.1.2 Background and aims to the pilot study

Lewis rats were chosen as both host and donor in the assessment of the functional capacity of transplanted metanephroi. This was due to one major reason: the Lewis rat is a highly inbred strain and so the physiological performance of transplanted metanephroi could be investigated without the risk of rejection. This meant that no tolerance inducing agents needed to be administered, nor was the host animal immunocompromised in any way. However, this choice of animal led to other problems. Lewis rats have been used in renal clearance studies far less often than other strains such as Sprague-Dawley and Wistar rats. Consequently, published data are relatively scarce and there is wide variation between studies. For example, renal blood flow has been reported in a range between 2.5-6.5 ml min^{-1} g kid wt^{-1}, whereas GFR has been reported to vary from 0.5-1.5 ml min^{-1} g kid wt^{-1} (Perico et al., 1992;

Churchill et al., 1993; Haylor et al., 1996; Dragun et al., 1998; Inman et al., 2002; Troncoso et al., 2005). Accordingly, it was necessary to establish baseline renal function in Lewis rats in our hands.

The aims of this study were two-fold. First, to determine baseline renal function measurements in the Lewis rat. Second, to establish the most appropriate methodological approach to take when attempting to make clearance measurements in transplanted metanephroi.

The most commonly used method involves the infusion of clearance markers in vehicle solution, typically saline, at a constant rate. Published infusion rates vary between 25-200 μl/min in adult rats (Walker et al., 1983; Garland et al., 1999; Hammad et al., 2000; Sahajpal and Ashton, 2003; Song et al., 2006). This provides constant delivery of clearance markers, resulting in stable plasma concentrations over time, which is an essential requirement of the clearance protocol. However, even at relatively low infusion rates, there is a tendency towards expansion of blood volume. An alternative approach developed at The University of Manchester to overcome this problem is that of euvolaemic fluid replacement (Burgess et al., 1993; Garland et al., 1999). This technique, known as servo-controlled fluid replacement, matches the rate of infusion of vehicle to spontaneous urine output. Clearance markers are delivered via a second, slow constant infusion at a rate less than spontaneous urine production (1 ml/hr). The advantage of this method is that blood volume is not expanded, however the disadvantage is that urine volumes are small.

The other factor to be considered was the approach to urine collection. As a host rat would have a both a native kidney and transplant connected to

the bladder, it was essential that urine from different sources could be collected separately. Hence the pilot study also sought to determine if bilateral ureter cannulation led to changes in UV, GFR, ERBF and electrolyte excretion rates compared with animals with a single bladder cannulation.

2.2 Materials and methods

All experiments described herein were performed under the authority of a Home Office Project Licence (PPL numbers 40/2717 and 40/2455).

2.2.1 Animals

The animals employed in this study were female Lewis rats (Charles River UK) which were housed in the Biological Services Unit (BSU) at the University of Manchester. Animals were maintained in a 12 hour light/dark cycle with free access to water and food (Bantin and Kingman rat and mouse expanded diet, Hull, UK). At time of clearance study, animals employed in the pilot study were approximately 8-12 weeks old and weighed between 200-230g. Animals with metanephric transplants were approximately 6-7 months old and weighed between 280-320g at time of clearance.

2.2.2 Pilot clearance study

Female adult Lewis rats (Charles River UK) were anaesthetised with Inactin (Sigma-Aldrich, UK), a sodium barbiturate (100 mg/kg *i.p*). Animals were maintained at an appropriate level of anaesthesia throughout the experiment by administration of 10 mg/kg Inactin *i.v.* when necessary. A midline incision was made in the neck of each animal and a tracheostomy was performed, using cauterisation (Downs Distributors Ltd, Auckland, NZ), to assist ventilation. The carotid artery was cannulated with polyethylene tubing (Smiths Medical, Kent, UK) and the cannula attached to a pressure transducer (Spectramed Inc, Ohio, USA) linked to a PowerLab recording system (AD Instruments Ltd, Oxfordshire). The jugular vein was cannulated for infusion of ^{3}H inulin (2 μCi/hr, Amersham Biosciences, UK) and para-aminohippuric acid (PAH, 3 mg/hr, Sigma-Aldrich, UK) in a 2.5% dextrose solution (Baxter Healthcare Ltd, Norfolk). (Depicted in Figure 2.1). Dextrose was infused at either a constant rate of 50 μl/min or at a variable rate matching spontaneous urine output (SERVO). This system was developed by a previous group in this laboratory (Burgess et al., 1993). Animals had either an indwelling bladder catheter, or both ureters canulated, for sampling purposes (see below).

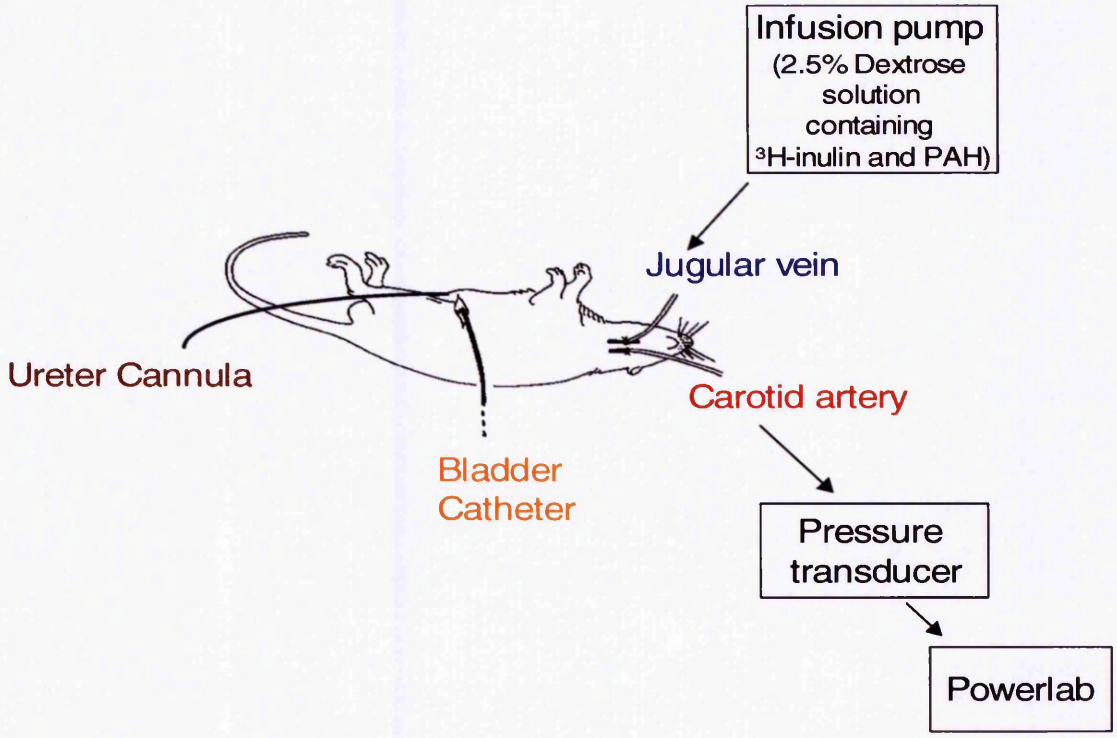

Figure 2.1. Standard clearance preparation. This approach was used in all animal groups with the exception that cannulation methods varied (either a single bladder catheter or two ureter cannulae). In the SERVO system, urine output was weighed constantly, with a computer automatically adjusting the volume of dextrose infused depending on the volume of urine output.

2.2.2.1 SERVO system: bladder vs ureters

The initial experiments focused on the use of the SERVO system to control infusion rates. It was decided that the urine would be collected in either of one of two ways. In the first animal group (n = 5), both ureters were cannulated to allow urine collection direct from each individual kidney (group US). Following a midline laparatomy, the intestines were displaced to one side and kept moist in cotton wool immersed in physiological saline.

The ureter was identified and a section proximal to the kidney dissected free from surrounding tissue. A small incision was made into the ureter and a cannula was introduced in a retrograde direction, towards the kidney. Once urine could be seen flowing through the cannula, the cannula was tied into place using Ethicon 5-0 suture (Ethicon Inc, Texas, USA). The process was repeated on the contralateral ureter and, following replacement of the intestines, the midline incision was sutured with an Ethicon 3-0 suture (Ethicon Inc, Texas). To assess whether this method resulted in improved urine flow rates (UV), another animal group (n = 6) was set up which had an indwelling bladder catheter only (group BS). A small incision was made towards the base of the abdomen along the mid-line. The bladder was identified and pulled through the incision. A 5-0 nylon ligature (Ethicon Inc, Texas) was placed around the bladder and a hole was cut in the bladder wall using a cauteriser (Downs Distributors Ltd, Auckland, NZ). Following cauterisation, the bladder was compressed to expel any urine and a catheter with a flared end was introduced into the bladder and tied firmly into place using the 5-0 ligature. The bladder was carefully placed back into the abdomen and a swab immersed in physiological saline was placed over the incision.

2.2.2.2 Constant infusion rate: bladder vs ureters

To determine which infusion method was the most suitable for use in animals with transplanted metanephroi, a second series of control animals was infused with 2.5% dextrose at a set infusion rate of 50 µl/min. This

group of rats was subdivided into those with a single bladder cannulation (group BC, $n = 7$), or those with both ureters cannulated (group UC, $n = 5$).

2.2.2.3 Timing of clearance study

Timing of the clearance study can be seen in Figure 2.2. Following a priming dose of ^{3}H-inulin (4 μCi, Amersham Biosciences, UK) and PAH (12mg, Sigma-Aldrich, UK), there was a 3 hr equilibration period. Urine samples were then collected every 15 min for the remaining 3 hr, with blood samples being taken at hourly intervals during the urine collection period. Blood samples were taken at the end of the experiment for the determination of haematocrit. All animals were euthanased at the end of the experiments, according to Schedule 1 procedures (Home Office, UK).

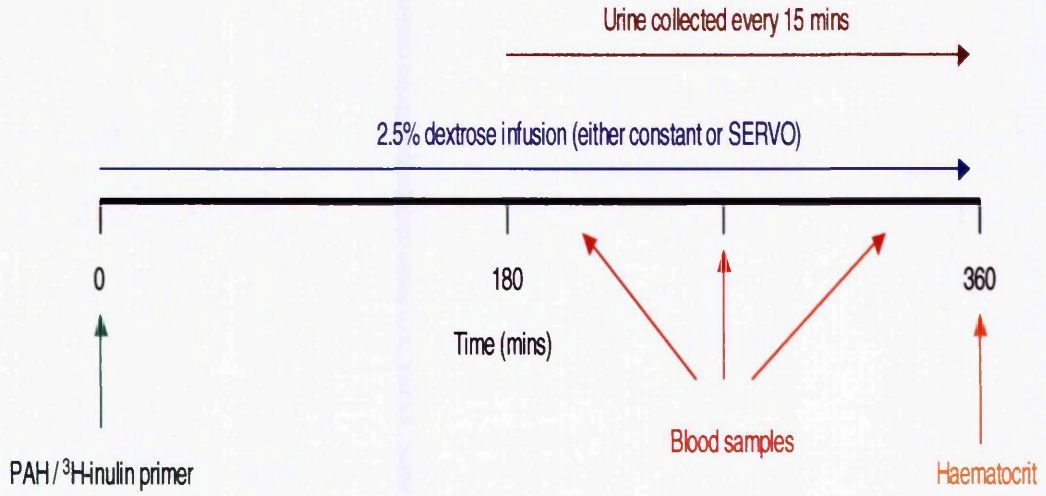

Figure 2.2. Timing of clearance study.

2.2.3 Animals with transplanted metanephroi

2.2.3.1 Transplantation of metanephroi and ureteroureterostomy

It was a condition of the UK Home Office licence (PPL 40/2455) that the transplantation of metanephroi and subsequent ureteroureterostomy was carried out by a trained surgeon, hence this aspect of the study was performed by Dr Marc Clancy. All other surgery (including clearance study preparative surgery) was carried out by the candidate.

Animals were prepared according to previously published protocols (Rogers et al., 1991). Embryonic day 15 (E15) rat embryos were obtained from time-mated Lewis rats (Charles River UK). Metanephroi were surgically dissected from E15 Lewis rat embryos using a dissecting microscope following Isoflurane gaseous anaesthesia (flow rate 1 L/min O_2, 2.5% Isoflurane) of the mother. Embryos were removed following a midline laparotomy of the mother and a midline laparotomy was performed on each embryo. The liver was removed allowing the metanephroi to be identified and dissected free of surrounding tissue. Metanephroi were stored in ice-cold DMEM media (Invitrogen Ltd, UK) in readiness for transplantation. Both the adult mother and embryos were humanely euthanased following this dissection, according to Schedule 1 methods.

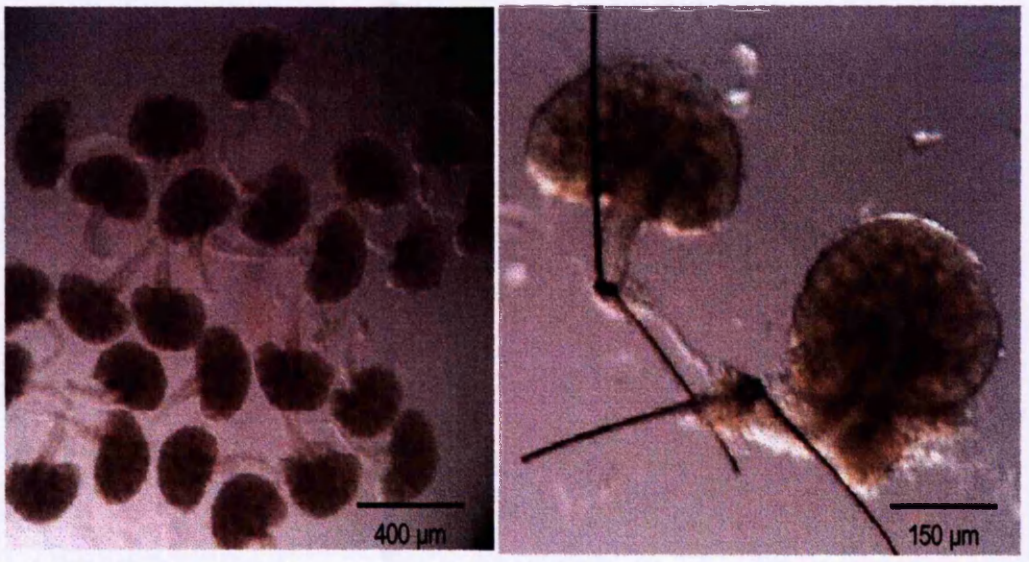

Figure 2.3. Images of E15 Lewis rat metanephroi. Isolated metanephroi are shown in the left-hand image. Two E15 (embryonic day 15) metanephroi have had their ureters tied with an 11-0 suture to aid identification in the right-hand image. Pictures courtesy of Intercytex Ltd.

Dissected metanephroi were incubated in ice cold DMEM media (Invitrogen Ltd, UK) containing the following growth factors, shown previously to enhance the growth of metanephroi *in vivo* and *in vitro* (Rogers et al, 1998; Rogers et al, 2001; Rogers & Hammerman, 2001): recombinant human insulin-like growth factor (IGF)-I 10^{-7}M (Upstate Biotech, Northern Ireland, UK), recombinant human IGF-II 10^{-7}M (Upstate Biotech, Northern Ireland, UK), recombinant human vascular endothelial growth factor 5µg/ml (Upstate Biotech, Northern Ireland, UK), recombinant human transforming growth factor alpha 10^{-8}M (Upstate Biotech, Northern Ireland, UK), recombinant human nerve growth factor 5µg/ml (R&D Systems, Oxon, UK), recombinant human fibroblast growth factor 5µg/ml (R&D Systems, Oxon, UK), recombinant human hepatocyte

growth factor 10^{-8}M (R&D Systems Oxon, UK), iron saturated transferrin 5µg/ml (Sigma-Aldrich, UK) corticotropin-releasing hormone 1µg/ml (Sigma-Aldrich, UK), retinoic acid 10^{-6}M (Sigma-Aldrich, UK), prostaglandin E1 25nM (Sigma-Aldrich, UK) and Tamm-Horsfall protein 1µg/ml (Biomedical Technologies). The growth factors had been previously shown to improve growth of transplanted metanephroi at these concentrations (Rogers et al., 1998; Rogers et al., 2001) and so these concentrations were used in the present study. Metanephroi, incubated on ice in this growth factor-rich media for a minimum of 1 hr, were implanted adjacent to the abdominal aorta of Isofluorane-anaesthetised (flow rate 1 L/min O_2, 2.5% Isoflurane) 6-week old adult female Lewis (host) rats following a midline laparotomy. A small incision was made into the fat adjacent to the aorta, proximal to the junction between the aorta and left renal artery, and metanephroi were inserted into this incision via a glass pipette. This aortic site has been shown previously to result in greater growth of the metanephroi by comparison with those transplanted onto the omentum (M.Clancy, M.Dilworth, D.Marshall, C.Bravery and N.Ashton, unpublished observations). A left nephrectomy was also performed on the host rat by removing the renal capsule and tying off the left renal artery and vein with an 11-0 suture (Ethicon Inc, Texas). The ureter was cleanly severed and kidney removed.

Animals were allowed to recover after surgery and an analgesic (0.02 mg Buprenorphine, Schering-Plough Ltd, Welwyn Garden City, UK) administered before the animals were returned to normal housing. All rats were then treated with the corticosteroid methylprednisolone (800

µg/kg/day s.c.), shown previously to improve the growth of transplanted metanephroi (M.Clancy, M.Dilworth, D.Marshall, C.Bravery and N.Ashton, unpublished observations), for a period of 21 days following transplantation. Approximately 3 to 4 weeks following transplantation of metanephroi, animals with transplants were once again anaesthetised with Isoflurane (flow rate 1 L/min O_2, 2.5% Isoflurane) and the free end of the ureter of the metanephros was connected via an interrupted 11-0 suture (Ethicon Inc, Texas) to the free end of the ureter previously connected to the host kidney that had been removed. This is a form of microvascular technique and is illustrated in figure 2.4B. Animals were allowed to recover and 0.02 mg Buprenorphin administered before the animals were returned to normal housing. Approximately 3 to 4 months following ureteroureterostomy animals were prepared for clearance study.

2.2.3.2 Surgery for clearance study (animals with transplants and controls)

Surgery for animals with transplants was exactly the same as for those undergoing clearance in the bladder vs ureter cannulation group (section 2.2.2) with two exceptions. All animals with metanephros transplants (n = 6) had their right ureter cannulated for collection of urine from the remaining host kidney, and a bladder cannulation for urine collection from the transplant. Dextrose (2.5 %) was infused at a constant rate of 40 µl/min in order to minimise extracellular fluid volume expansion. This rate was chosen on the basis of previous work in this laboratory (M.Clancy, D.Marshall and C.Bravery, unpublished observations), which has shown

that anaesthetised rats with one native kidney are able to clear this volume of fluid without a significant change in haematocrit.

Control animals (n = 5) were prepared in the same manner as animals with transplants, but both native kidneys were left intact. Hence, urine produced by the right native kidney was collected via the ureter cannula and urine from the left native kidney was collected via the indwelling bladder catheter.

2.2.3.3 Obtaining metanephric cyst fluid

Approximately 3 weeks after transplantation of metanephroi, a laparotomy was performed under Isoflurane anaesthesia (flow rate 1 L/min O_2, 2.5% Isoflurane). Re-exploration of the transplanted tissue sometimes revealed that a fluid-filled cyst had developed around the free-end of the metanephric ureter (Figure 2.4). To determine whether this fluid contained urea, as a marker of urinary concentration, cyst fluid was aspirated via a sterile syringe and stored at -20 °C prior to analysis. Animals were euthanased by a Schedule 1 method following explantation of the transplanted metanephroi, which were either placed in 4% paraformaldehyde (PFA) for histology or liquid nitrogen for later analysis. Serum, urine from the metanephros and urine from the native kidney were all collected from animals with transplanted metanephroi following clearance study. Serum was collected via the carotid artery cannula, prior to euthanisation, whilst urine from the metanephros was collected following the 3-hour collection period. Urine from the native kidney was

collected during a 15-minute period in the clearance study. Samples were stored at -20 °C until analysed for urea content.

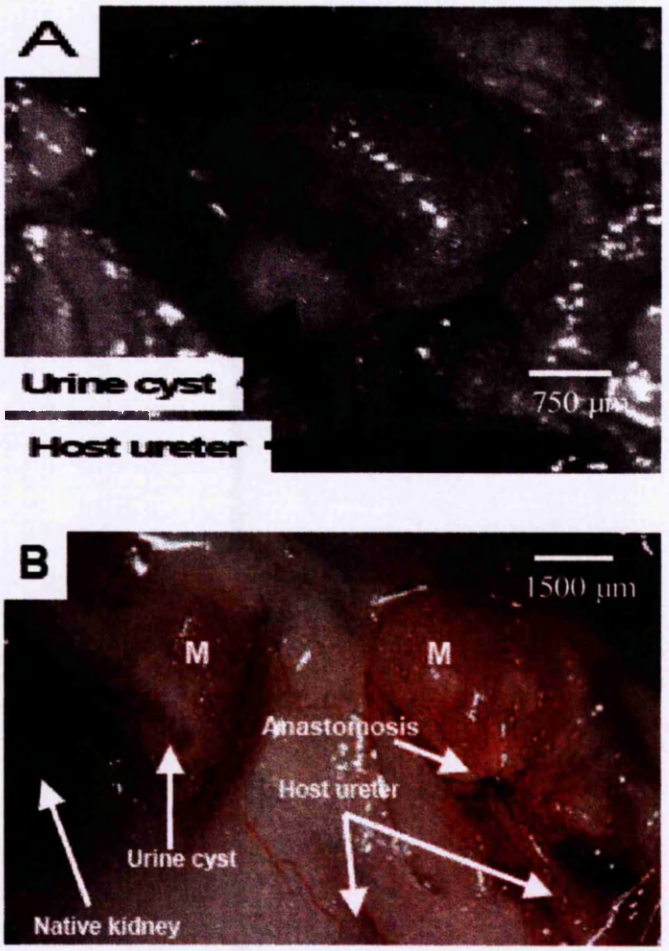

Figure 2.4. Images of *in situ* transplanted metanephroi. (A) Image of a metanephros 19 days after transplantation to the left renal vessel of an adult female Lewis rat. Note the presence of a urine cyst. (B) Image of 2 metanephroi transplanted onto the omentum of an adult female Lewis rat. Note the presence of a urine cyst on the transplant on the left. Also visible is the connection of host ureter to metanephric ureter on the right. Both images courtesy of Intercytex Ltd.

2.2.4 Analysis of urine and plasma samples

2.2.4.1 ^{3}H Inulin

The radioactivity of urine and plasma samples was measured using a 1900CA Tri-Carb Liquid Scintillation Analyser (Canberra Packard β-counter, Austria), using optiphase scintillant (Fisher Chemicals, Loughborough, U.K.). 10 µl of urine or plasma were aliquotted into 4 mL of scintillation solution and counted for 5 minutes.

2.2.4.2 Measurement of PAH

The PAH concentrations for all urine and plasma samples were determined by colorimetry. Prior to analysis, plasma samples were deproteinised by adding 400 µl $ZnSO_4H_2O$ (Sigma-Aldrich, UK) to 50 µl plasma, vortexing using a Genie 2 vortex (S.H. Scientific, Northumberland), then adding 50 µl 0.1 M NaOH (VWR International, Poole), vortexing and centrifuging after 1 hour in a micro centrifugette 4214 (Camlab, Cambridge). Samples were diluted as follows prior to analysis; 5 µl urine + 495 µl H_2O or 400 µl plasma + 100 µl H_2O. To each 500 µl sample, 400 µl 1 M HCl (VWR International, Poole) and 100 µl 0.01M $NaNO_2$ (Sigma-Aldrich, UK) were added. The solution was vortexed then 100 µl 0.05M ammonium sulphamate (Sigma-Aldrich, UK) and 100 µl 0.01M 1-Napthylethylenediamine-diHCl (Sigma-Aldrich, UK) were added. The solution was vortexed and left for 15-20 minutes. PAH standards were

prepared (0, 0.1, 0.5, 1.0, 5.0 mg %) from a 10 mg % acid PAH stock solution (Sigma-Aldrich, UK) and a standard curve was constructed. Absorption was read at 540nm using a Beckman DU-530 UV/VIS spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA.) Urine and plasma sample values were then read off the curve and corrected for dilution.

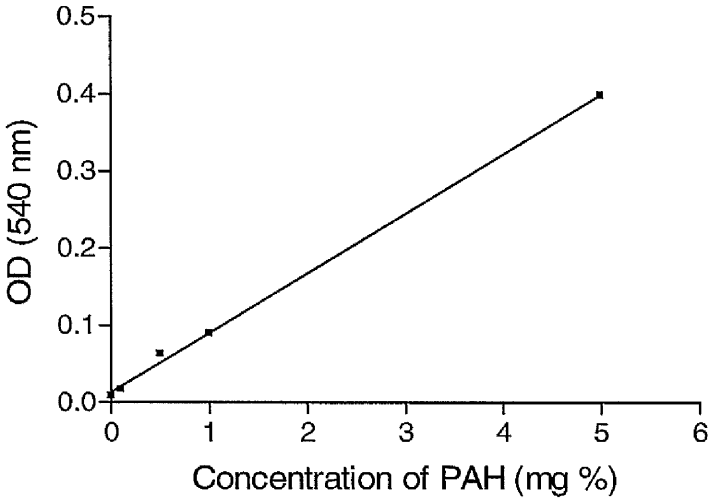

Figure 2.5. Example of a PAH standard curve. Standard curve produced from PAH (Para-aminohippuric acid) standards prepared at 0, 0.1, 0.5, 1 and 5 mg %.

2.2.4.3 Calculation of clearance and excretion rates

^{3}H inulin (GFR), PAH (ERPF), electrolyte and osmolar clearances were calculated using the equation

$C_x = U_x V/P_x$

where C = clearance, U = concentration in urine, V = flow rate and P = concentration in plasma.

Electrolyte and osmolar excretion rates were calculated as follows:

$U_xV = U_x x (UV/1000)$

Fractional excretion of electrolytes were calculated as follows:

$FE_x = (U_xV / (Px/GFR)) *100$

Effective Renal Plasma Flow (ERPF) =

$$\frac{\text{urine flow rate (ml/min) x urine PAH (µg/ml)}}{\text{plasma PAH (µg/ml)}}$$

Effective renal blood flow (ERBF) was calculated from:

ERBF = ERPF/ 1.0 – haematocrit

Renal vascular resistance (RVR) was calculated from:

RVR = MAP/ERBF

Where MAP = mean arterial pressure

2.2.4.4 Mean arterial pressure

Arterial pressure was measured directly via the carotid artery throughout the experimental period and recorded by the PowerLab software (AD Instruments Ltd, Oxfordshire). Mean arterial pressure (MAP) was calculated as diastolic blood pressure (DBP) + 1/3 of pulse pressure.

2.2.4.5 Measurement of osmolality

Osmolality of urine and plasma samples was determined by freezing point depression using a Roebling osmometer (Camlab Ltd, Cambridge, UK). The osmometer was standardised with a solution of known osmolality (300 mOsm/kg H_2O) (Camlab Ltd, Cambridge, UK). 50 µl of sample was used for all measurements.

2.2.4.6 Measurement of sodium and potassium

Sodium and potassium concentrations in urine and plasma samples (75 µl) were measured using a Corning 480 Flame Photometer (Ciba Corning Diagnostics Ltd, Essex, UK). A 3 M lithium internal standard was used and the flame photometer was standardised for urine (160 mmol/L NaCl, 80 mmol/L KCl) or plasma (140 mmol/L NaCl, 5 mmol/L KCl) using Corning MultiCal vials. All samples were undiluted.

2.2.4.7 Analysis of urea concentrations

The urea concentrations of serum, urine (produced from native kidneys and transplanted metanephroi) and cyst fluid were determined by colorimetry. An Enzymatic Urea Nitrogen clinical testing kit was used (Stanbio laboratories, Texas, US) which employed a modified version of the Berthelot reaction (Tabacco et al., 1979). Briefly, urine and cyst fluid samples were diluted as follows; 10 µl sample + 990 µl H_2O. Plasma samples were undiluted. To 10 µl of each sample and a urea nitrogen

standard (known concentration of 30 mg/dL), 1 mL of enzyme reagent (containing phosphate buffer at 120 mmol/L, sodium salicyclate at 60 mmol/L, sodium nitroprusside at 3.2 mmol/L, EDTA at 1 mmol/L and urease at 10 KU/L) was added and the solution vortexed. Following a 10 minute incubation at room temperature (RT), 1 mL of colour reagent (containing sodium hydroxide at 130 mmol/L and sodium hypochloride at 6 mmol/L) was added. The solution was vortexed and incubated for 10 minutes at RT. Absorption was read at 600 nm using a Beckman DU-530 UV/VIS spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA). Concentrations were calculated according to the following formula:

$$\text{Urea Nitrogen (mg/dL)} = \frac{\text{absorption of unknown sample}}{\text{absorption of standard}} \times 30 \text{ (mg/dL)}$$

To achieve the actual urea concentration for urine and cyst samples, the concentration derived from the above formula was multiplied by the dilution factor. It is acknowledged that the above calculation of urea calculation did not take into account the concentration of 'free ammonia' within the sample.

2.2.5 Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis was carried out using the Statistical Package for Social Sciences version 13.0 (SPSS 13.0 for Windows). A two-way ANOVA design was used to determine the

effect of cannulation method (bladder vs ureter) and infusion method (constant vs SERVO) in the pilot study. A one-way ANOVA was used to compare animals with transplanted metanephroi with control animals with the exception of mean arterial pressure, body and kidney weights and haematocrit values, where unpaired t-tests were employed. A test of homogeneity of variances showed that all data, with the exception of mean arterial pressure (pilot study and transplant study) and potassium fractional excretion (pilot study), were not normally distributed, so the ANOVAs were performed following $\log_{10}$ transformation of data. Statistical significance was set at the 5 % level.

2.3 Results

2.3.1 Pilot in vivo *clearance study*

The following data represent the outcome of 4 different treatment groups: bladder cannulated controls infused via the SERVO system (BS, n = 6), ureter cannulated controls infused via the SERVO system (US, n = 5), bladder cannulated controls infused via the constant rate infusion system (BC, n = 7) and ureter cannulated controls infused via the constant rate infusion system (UC, n = 5).

Animal data from the pilot study can be seen in table 2.1. There was no statistical difference in body weights between the BC group and BS (P = 0.117), UC (P = 0.979) or US groups (P = 0.180). Neither was there any difference between the BS group and UC (P = 0.303) and US groups (P = 0.999). One-way ANOVA also failed to show any difference between UC and US groups (P = 0.399) for body weight.

Similarly, the BC group showed no difference in combined kidney weight compared with BS (P = 0.999), UC (P = 0.970) and US groups (P = 0.881). Kidney weights in the BS group were comparable to both the UC (P = 0.990) and US groups (P = 0.835). Likewise, kidney weights in the US and UC groups did not differ (P = 0.697).

This pattern was also observed for kidney weight expressed as percentage of bodyweight (KW/BW %) with the BC group comparable to BS (P =0.963), UC (P = 0.984) and US groups (P = 0.579). The BS group showed a KW/BW % value not significantly different to either UC

($P = 0.863$) or US group ($P = 0.848$). UC and US groups failed to show a significant difference ($P = 0.444$).

Haematocrit values were fairly consistent across all groups. Values for the BC group were comparable with BS ($P = 0.588$), UC ($P = 1.000$) and US groups ($P = 0.998$). Likewise, haematocrit values failed to differ between the BS group and the UC ($P = 0.674$) and US groups ($P = 0.549$). The haematocrits of UC and US groups were also comparable ($P = 0.997$).

Plasma osmolality (P_{osm}) showed a statistical difference between the BC group compared with BS ($P = 0.044$), UC ($P = 0.005$) and US groups ($P = 0.020$). This difference was also seen when comparing the BS group with both the UC and US groups (both $P = 0.001$). One-way ANOVA failed to detect a difference between the UC and US groups for P_{osm} ($P = 0.968$).

Sodium concentration in plasma (P_{Na}) was not significantly different in the BC group compared with the BS ($P = 0.224$), UC ($P = 0.794$) and US groups ($P = 0.538$). In contrast, P_{Na} was significantly higher in the BS group compared with both the UC ($P = 0.038$) and US groups ($P = 0.012$). There was no difference between UC and US groups for P_{Na} ($P = 0.977$).

Potassium concentration in plasma (P_K) was not statistically different between the BC group and BS ($P = 0.651$), UC ($P = 0.910$) and US groups ($P = 0.308$). Likewise, there was no difference in P_K between the BS group compared with both the UC ($P = 0.299$) and US groups ($P = 0.920$). There was also no statistical difference in P_K between the UC and US groups ($P = 0.109$).

	BC (n = 7)	BS (n = 6)	UC (n = 5)	US (n = 5)
Body weight (g)	205 ± 2	215 ± 4	207 ± 1	214 ± 2
Combined kidney weight (g)	1.76 ± 0.08	1.77 ± 0.05	1.84 ± 0.18	1.66 ± 0.06
Kidney weight/ Body weight (%)	0.86 ± 0.04	0.83 ± 0.03	0.89 ± 0.08	0.78 ± 0.03
Haematocrit (%)	43.7 ± 1.9	40.3 ± 1.7	43.6 ± 1.9	44.2 ± 2.3
P_{osm} (mOsmol/kg)	274 ± 2*	294 ± 7	252 ± 6*#	255 ± 5*#
P_{Na} (mmol/L)	135 ± 1	142 ± 3	132 ± 1*	131 ± 4*
P_K (mmol/L)	4.27 ± 0.07	4.54 ± 0.14	4.14 ± 0.11	4.79 ± 0.3

Table 2.1. Baseline data from pilot clearance studies. Groups are as follows; bladder cannulation with constant rate infusion (BC), bladder cannulation with SERVO infusion system (BS), ureters cannulated with constant infusion (UC) and ureters cannulated with SERVO system (US). P_{osm} = osmolality within plasma, P_{Na} = sodium concentration within plasma, P_K = potassium concentration within plasma. $P < 0.05$ was deemed significant. # $P < 0.05$ compared to BC group, * $P < 0.05$ compared to BS group.

For all of the graphs that follow (Figs 2.6-2.12), the values shown are a mean of the 3 hour collection period (hours 4-6 of the clearance protocol).

2.3.1.1 *Mean arterial pressure*

Mean arterial pressure (MAP) is shown in figure 2.6. Two-way ANOVA showed that neither choice of cannulation method (ureters or bladder, $P = 0.594$) nor infusion method (SERVO or constant rate, $P = 0.095$) had an effect on the MAP of the animals during clearance. MAP was stable throughout the six-hour duration of the clearance study.

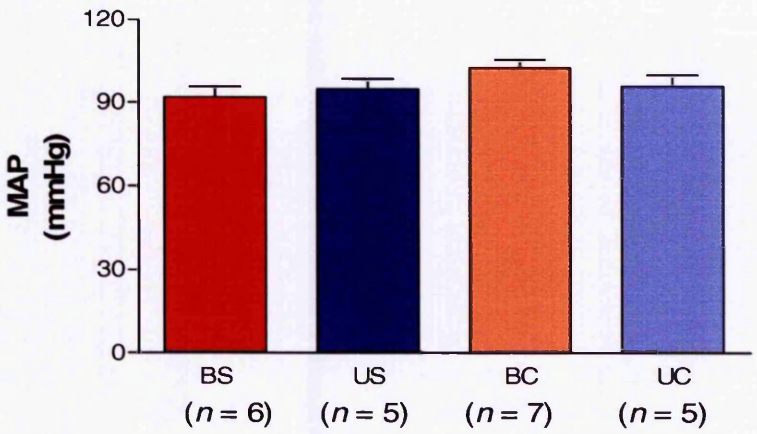

Figure 2.6. Mean arterial pressure in control animals. Measured over a 3-hour period. Groups are as follows; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation $P = 0.594$, SERVO vs constant $P = 0.095$, interaction $P = 0.177$.

2.3.1.2 *Effective renal blood flow*

The effective renal blood flow (figure 2.7) measured in rats with bladder cannulations was significantly lower (P = 0.001) than that in animals with both ureters cannulated. This was most noticeable in the BS group which had an ERBF 50% lower than that of ureter-cannulated matched controls (group US). Infusion type also had a significant effect, with SERVO control animals having lower renal blood flow values than animals on a constant infusion rate (P = 0.043). This difference appears most noticeable when comparing the two groups with indwelling bladder catheters, which accounts for the significant interaction between infusion type and cannulation type (P = 0.001) detected in the ANOVA.

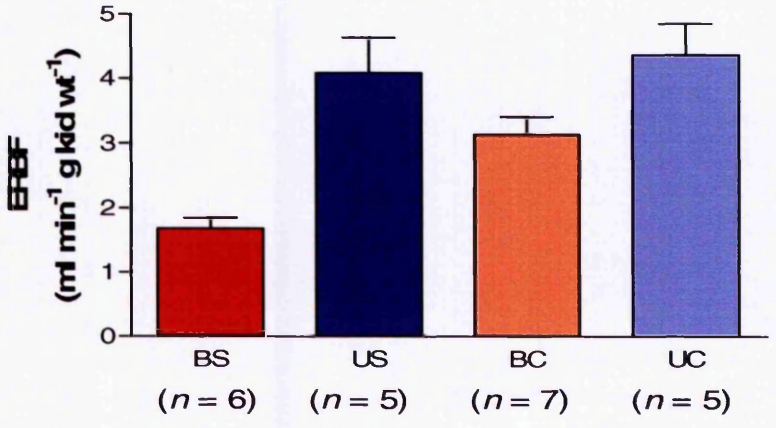

Figure 2.7. Effective renal blood flow in control animals. Groups are as follows; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation P = 0.001, SERVO vs constant P = 0.043, interaction P = 0.001.

2.3.1.3 *Glomerular filtration rate*

A similar pattern was also observed for GFR (figure 2.8). The method of cannulation significantly altered GFR measurements, with animals having indwelling bladder catheters showing reduced GFRs compared with ureter cannulated controls ($P = 0.001$). This was seen in both BS and BC groups, with GFR values being 50% lower than their corresponding ureter control group (US and UC control groups respectively). Infusion method also contributed to the variability of GFR measurements with SERVO animals giving lower estimates than animals on a constant infusion rate ($P = 0.001$). There was a significant interaction ($P = 0.011$) between cannulation and infusion methods.

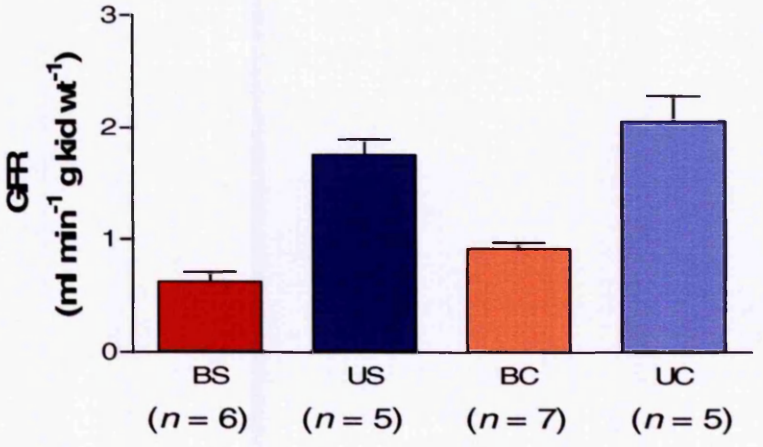

Figure 2.8. Glomerular filtration rate in control animals. Groups are as follows; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation $P = 0.001$, SERVO vs constant $P = 0.001$, interaction $P = 0.011$.

2.3.1.4 *Urine flow rate*

Urine flow rate (figure 2.9) was reduced in both bladder cannulated (P = 0.001) and SERVO control (P = 0.001) animals compared to ureter cannulated controls and rats infused at a constant rate, respectively. The ratio between UV in ureter controls and their respective bladder controls (US to BS and UC to BC) was remarkably similar at 2.6:1. In contrast to the measures of GFR and ERBF, there was no interaction between the two parameters tested for urine flow rate (P = 0.734).

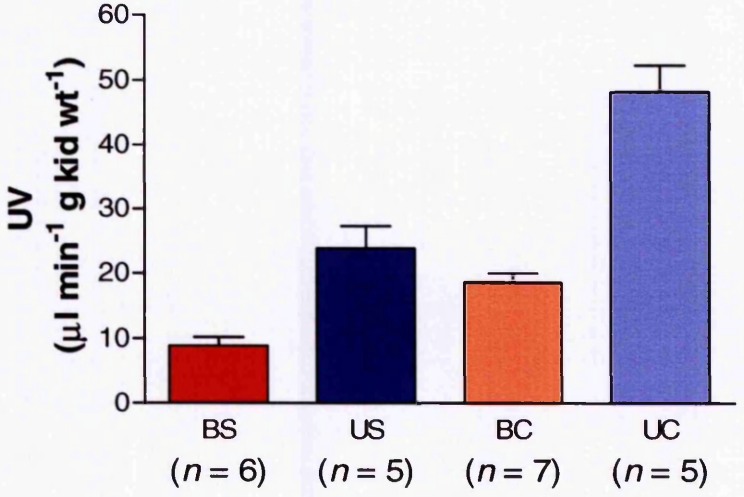

Figure 2.9. Urine flow rate in control animals. Groups are as follows; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation P = 0.001, SERVO vs constant P = 0.001, interaction P = 0.734.

2.3.1.5 Osmolar excretion and clearance rates

This general pattern of increased urinary output in ureter-cannulated and constant infusion controls was repeated for both osmolar excretion and clearance rates (figures 2.10A and B). Cannulation technique had a marked effect on both of these parameters, with significantly reduced rates for both excretion and clearance rates in animals with a single bladder cannulation (BS and BC, both $P = 0.001$). Choice of infusion technique appeared to have less effect on either osmolar excretion or clearance, though in both of these measurements the differences seen were still significant ($P = 0.049$ for osmolar excretion, $P = 0.005$ for osmolar clearance). Two-way ANOVA showed that these factors exerted influences on excretion and clearance rates independently of each other (interaction $P = 0.364$ for osmolar excretion, $P = 0.189$ for osmolar clearance).

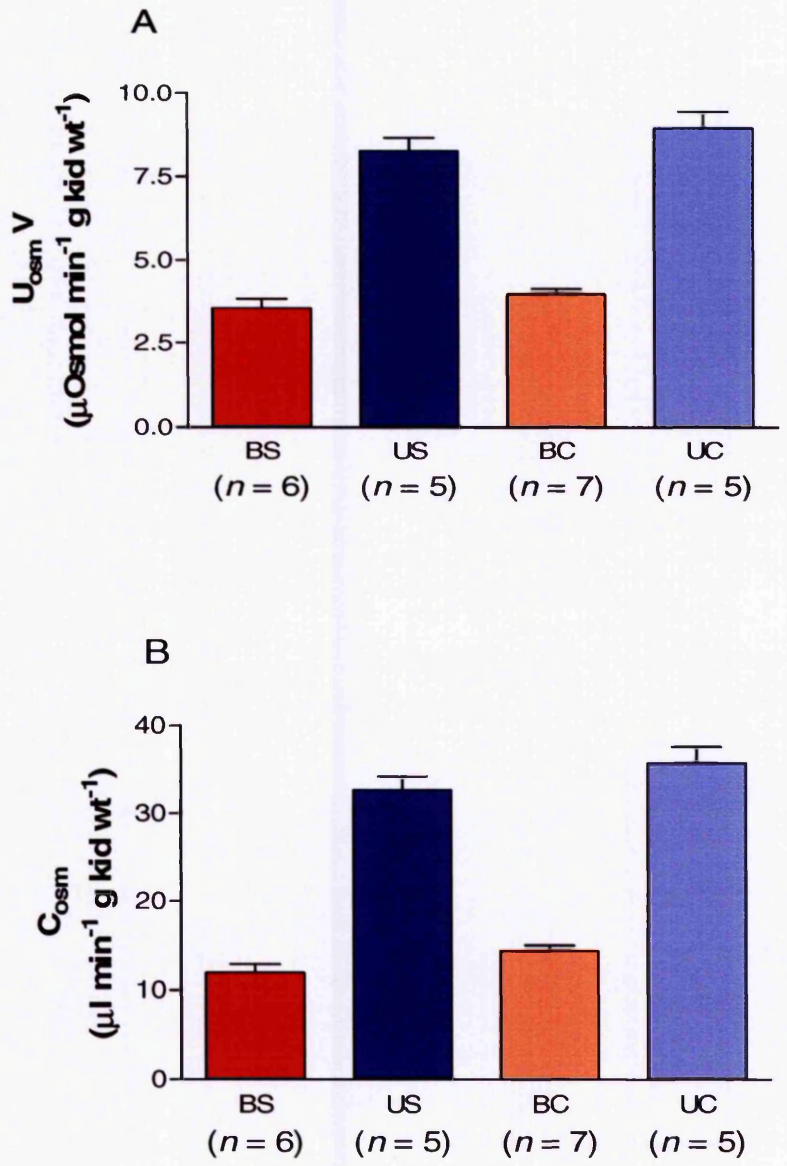

Figure 2.10. Osmolar excretion and clearance rates in control animals. Osmolar excretion rates (U_{osm} V) (A) and osmolar clearance rates (C_{osm}) (B) in; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation $P = 0.001$ for (A) and (B), SERVO vs constant $P = 0.049$ for (A) and $P = 0.005$ for (B), interaction $P = 0.364$ for (A) and $P = 0.189$ for (B).

2.3.1.6 Renal sodium handling

In order to assess renal sodium handling by the kidneys of control rats, sodium excretion and clearance rates were measured, as well as the fractional excretion of sodium (figures 2.11A,B and C). It was observed that both cannulation type and infusion technique contributed to the variation in sodium excretion and clearance rates. Animals undergoing bladder cannulations had statistically lower sodium excretion and clearance rates (both $P = 0.001$) than rats in the ureter-cannulated groups. Likewise, SERVO-infused controls had statistically lower excretion and clearance rates of sodium (both $P = 0.001$) than animals on a constant infusion. There was no evidence of an interaction between cannulation type and infusion technique for either sodium excretion ($P = 0.123$) or clearance rates (both $P = 0.073$). Both cannulation type ($P = 0.001$) and infusion protocol ($P = 0.001$) affected the fractional excretion of sodium (FE_{Na}). Bladder-cannulated controls and SERVO-infused had lower FE_{Na} than their respective counterparts. There was also a significant interaction between cannulation technique and infusion method for FE_{Na} ($P = 0.036$).

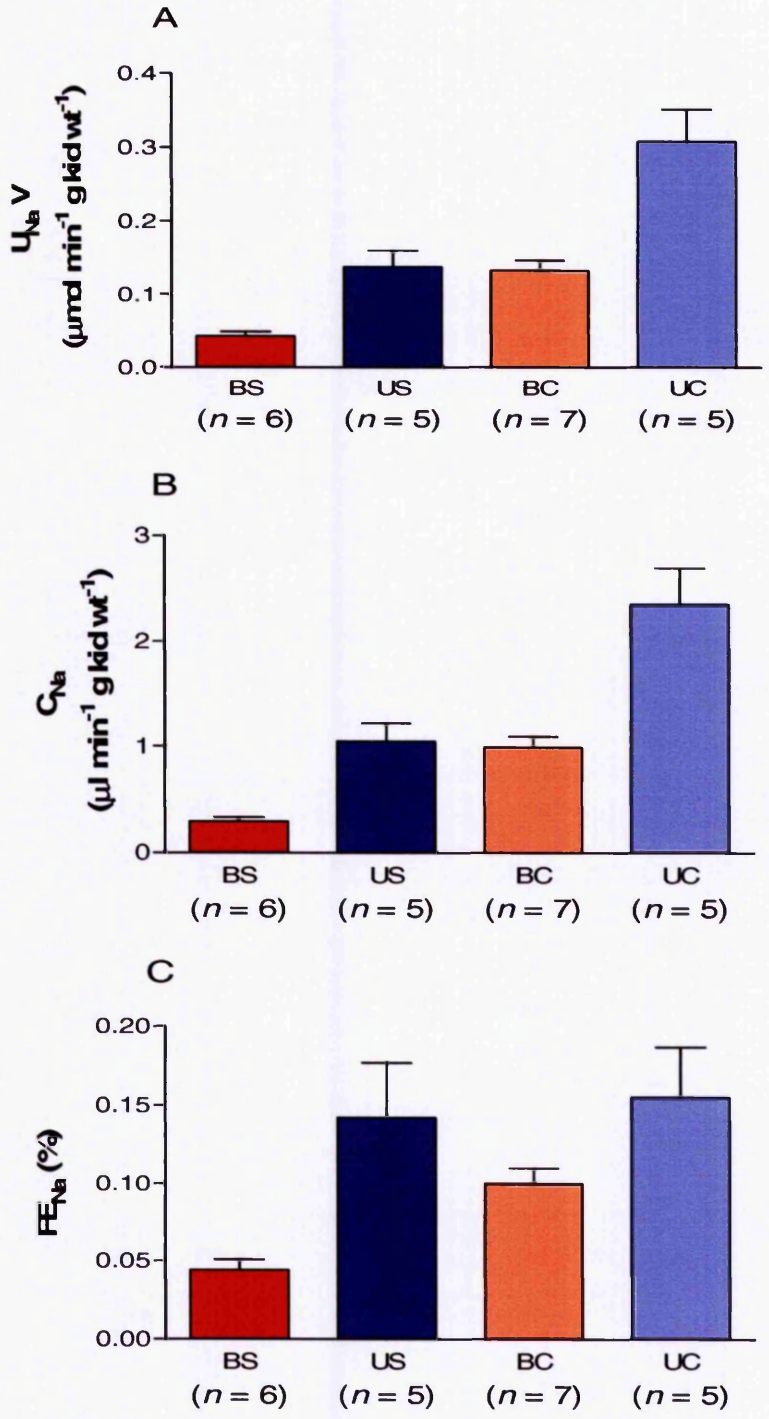

Figure 2.11. Renal sodium handling in control animals. Sodium excretion rates ($U_{Na}V$)(A), sodium clearance rates (C_{Na})(B) and sodium fractional excretion (FE_{Na})(C) in control groups. Groups are as follows; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation $P = 0.001$ for (A), (B) and (C), SERVO vs constant $P = 0.001$ for (A), (B) and (C), interaction $P = 0.123$ for (A), $P = 0.073$ for (B) and $P = 0.036$ for (C).

2.3.1.7 Renal potassium handling

Potassium excretion and clearance rates (2.12 A and B) tended to show a similar pattern to that observed for sodium handling, with bladder-cannulated controls having lower potassium excretion (P = 0.001) and clearance rates (P = 0.001) than those animals with their ureters cannulated. Likewise, animals undergoing infusion at a constant rate had significantly higher potassium excretion (P = 0.001) and clearance rates (P = 0.001) compared with the SERVO group. There was no significant interaction between cannulation and infusion methods for either potassium excretion (P = 0.207) or clearance (P = 0.415). The results for the fractional excretion of potassium (FE_K) were in complete contrast to those for potassium excretion and clearance rates, with no statistical effect of either cannulation type (P = 0.861) or choice of infusion protocol (P = 0.057) on FE_K (figure 2.12C).

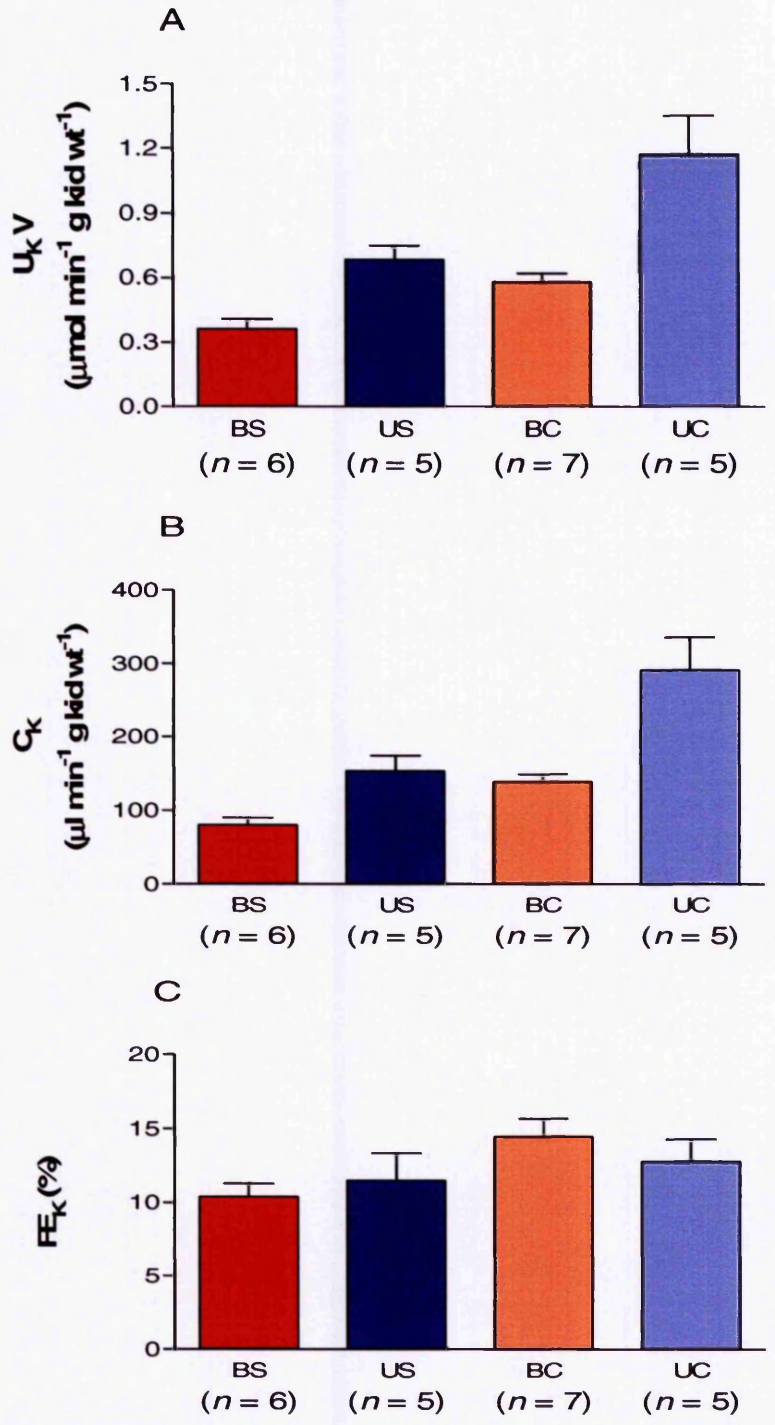

Figure 2.12. Renal potassium handling in control animals. Potassium excretion rates (U_KV)(A), potassium clearance rates (C_K)(B) and potassium fractional excretion (FE_K)(C) in control groups; bladder cannulation with SERVO infusion system (BS), ureters cannulated with SERVO system (US), bladder cannulation with constant rate infusion (BC) and ureters cannulated with constant infusion (UC). All data are presented as mean ± S.E.M. Statistical analysis was by two-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant. Bladder vs Ureter cannulation $P = 0.001$ for (A) and (B), $P = 0.861$ for (C), SERVO vs constant $P = 0.001$ for (A) and (B), $P = 0.057$ for (C), interaction $P = 0.207$ for (A), $P = 0.415$ for (B) and $P = 0.378$ for (C).

2.3.2 Animals with transplanted metanephroi

At this point, it is useful to re-iterate the infusion and cannulation techniques that were employed in this part of the study. Dextrose (2.5%) was infused at a constant rate of 40 µl/min whilst in both the transplant (Tx) animals and control animals (native renal tissue only) the right ureter was cannulated for collection of urine from the right native kidney. The bladder was also cannulated for collection of urine from the transplanted metanephros (Tx) and left native kidney (controls) respectively.

Animal data from the clearance study can be seen in table 2.2. Animals with transplants at the time of clearance study were significantly heavier than control animals ($P = 0.002$). As expected, transplanted metanephroi were significantly lighter than the left kidneys of control animals ($P = 0.001$). Interestingly, right kidney weight was greater in animals with transplants compared to right kidneys of control animals, both when expressed as raw weights ($P = 0.001$) and as a percentage of body weight ($P = 0.002$). Mean arterial pressure ($P = 0.449$) and haematocrit values ($P = 0.815$) showed no differences between groups.

	Tx animals (n = 6)	Control animals (n = 5)
Days post transplant	179 ± 35	N/A
Body weight (g)	303 ± 15*	227 ± 7
Left kidney weight (g)	0.05 ± 0.01* (Metanephros)	0.87 ± 0.03
Right kidney weight (g)	1.44 ± 0.07*	0.89 ± 0.07
Right kidney weight / body weight (%)	0.47 ± 0.01*	0.38 ± 0.02
Mean arterial pressure (mm Hg)	112 ± 3	109 ± 3
Haematocrit (%)	42.5 ± 2.2	43.2 ± 1.7

Table 2.2. Baseline data in transplanted metanephroi and control animals. Data shows number of days post-transplant when clearance measurements were performed in animals with transplants (Tx animals). Control animals had no transplant renal tissue but underwent the same clearance protocol as Tx animals. Body weights shown are from Tx animals at 6-7 months of age and control animals at 3 months of age. Also shown are kidney weights at time of clearance study along with mean arterial pressure and haematocrit values. All data are presented as mean ± S.E.M. Statistical analysis was by unpaired t-test. $P < 0.05$ was deemed significant. *$P < 0.05$ compared to controls.

2.3.2.1 *Effective renal blood flow*

Transplanted metanephroi had a statistically lower ERBF (figure 2.13) compared with the remaining native kidney of the host animal (P = 0.001) and the left and right kidneys of control animals (P = 0.001). ERBF from metanephroi was around 5% of that of the host's remaining native kidney. Blood flow in native kidneys was significantly higher than that of the left kidneys of control rats (P = 0.011), but not that of right kidneys (P = 0.221).

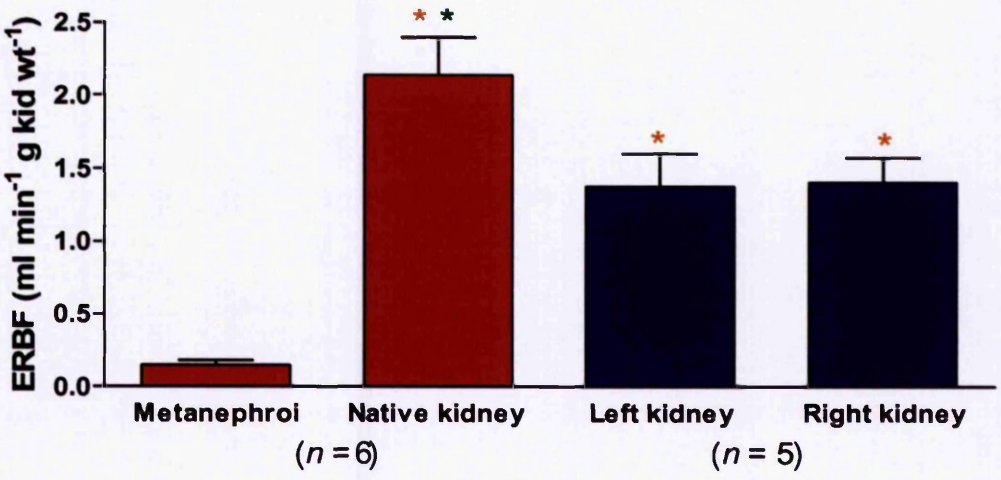

Figure 2.13. Effective renal blood flow in transplanted metanephroi and controls. Effective renal blood flow measured in transplanted metanephroi and remaining right native kidney of animals with transplants (red bars), and left and right kidneys of control animals (blue bars). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA, following $\log_{10}$ transformation of data, and Tukeys post-hoc test. $P < 0.05$ was deemed significant. * P = 0.001 compared to metanephroi. * P = 0.011 compared to left kidney of control animals.

2.3.2.2 *Renal vascular resistance*

Transplanted metanephroi had markedly higher vascular resistance (figure 2.14) compared with both the host's native renal tissue ($P = 0.001$) and the left and right kidneys of control animals ($P = 0.001$). Vascular resistance was not statistically different between control kidneys and native renal tissue of the animals with transplants ($P = 0.701$ vs left kidney, $P = 0.448$ vs right kidney).

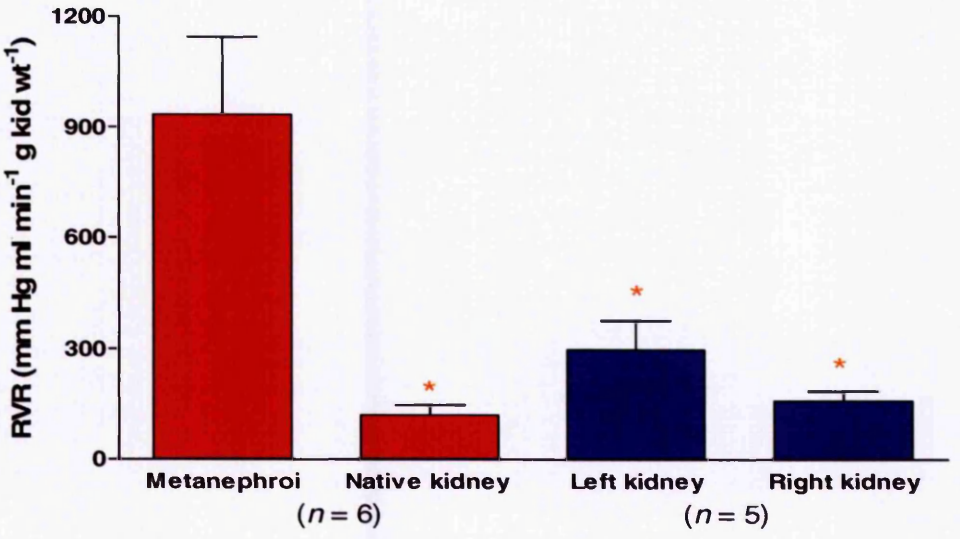

Figure 2.14. Renal vascular resistance in transplanted metanephroi and controls. Renal vascular resistance measured in transplanted metanephroi and remaining right native kidney of animals with transplants (red bars), and left and right kidneys of control animals (blue bars). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA, following $\log_{10}$ transformation of data, and Tukeys post-hoc test. $P < 0.05$ was deemed significant. * P = 0.001 compared to metanephroi.

2.3.2.3 *Glomerular filtration rate*

GFR (figure 2.15) was markedly reduced in transplanted metanephroi by comparison with all other kidneys (P = 0.001). There was no statistically significant difference between the host's native kidney and either the left (P = 0.988) or right control kidneys (P = 0.750) with values fairly consistent at around 0.8 ml min^{-1} g kidney $weight^{-1}$.

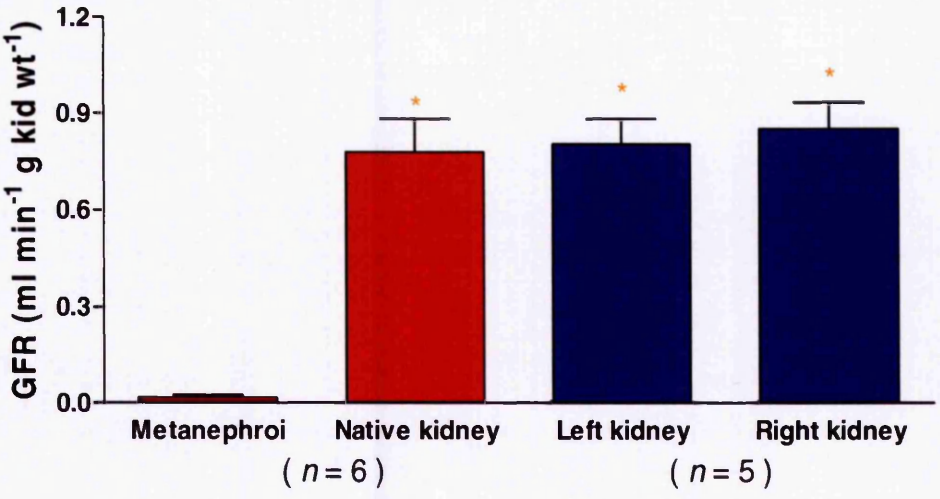

Figure 2.15. Glomerular filtration rate in transplanted metanephroi and controls. Glomerular filtration rate measured in transplanted metanephroi and remaining right native kidney of animals with transplants (red bars), and left and right kidneys of control animals (blue bars). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA, following log_{10} transformation of data, and Tukeys post-hoc test. $P < 0.05$ was deemed significant. * P = 0.001 compared to metanephroi.

2.3.2.4 *Urine flow rate*

Urine flow rate (figure 2.16) from the transplanted metanephroi, when standardised to kidney weight, tended to be lower than that of the adult kidneys, but this difference was not statistically significant. Urine flow rates were comparable with native kidney (P = 0.913) and left (P = 0.317) and right (P = 0.527) control kidneys. Flow rates from native kidney and left (P = 0.778) and right (P = 0.945) control kidneys were not statistically different.

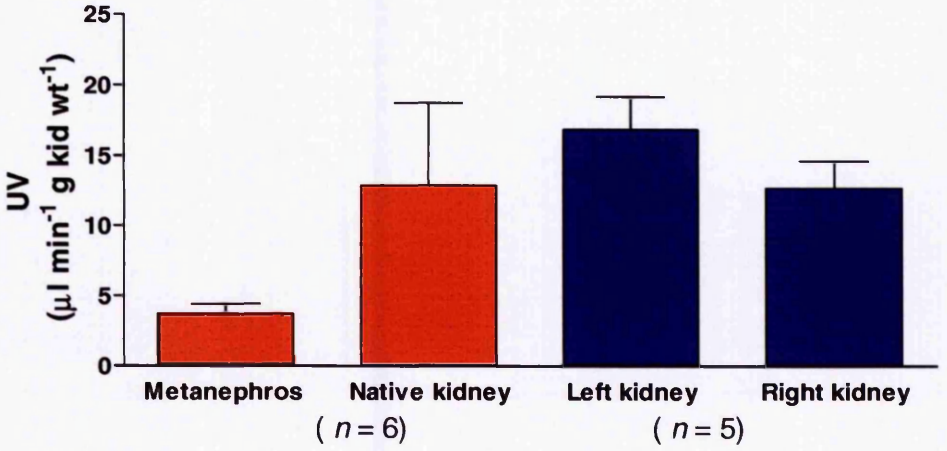

Figure 2.16. Urine flow rates in transplanted metanephroi and controls. Urine flow rates measured in transplanted metanephroi and remaining right native kidney of animals with transplants (red bars), and left and right kidneys of control animals (blue bars). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA, following $\log_{10}$ transformation of data, and Tukeys post-hoc test. $P < 0.05$ was deemed significant.

2.3.2.5 *Urine:Plasma ^{3}H-Inulin ratios*

The ratio of urine:plasma for ^{3}H-inulin was calculated as a marker of urine concentration by the metanephroi. Ratios of urine:plasma ^{3}H-inulin (figure 2.17) were significantly lower in transplanted metanephroi compared with the host's native kidney and left and right control animals (all $P = 0.001$). Ratios between host's native kidney and left ($P = 0.864$) and right ($P = 0.465$) control kidneys were not statistically different.

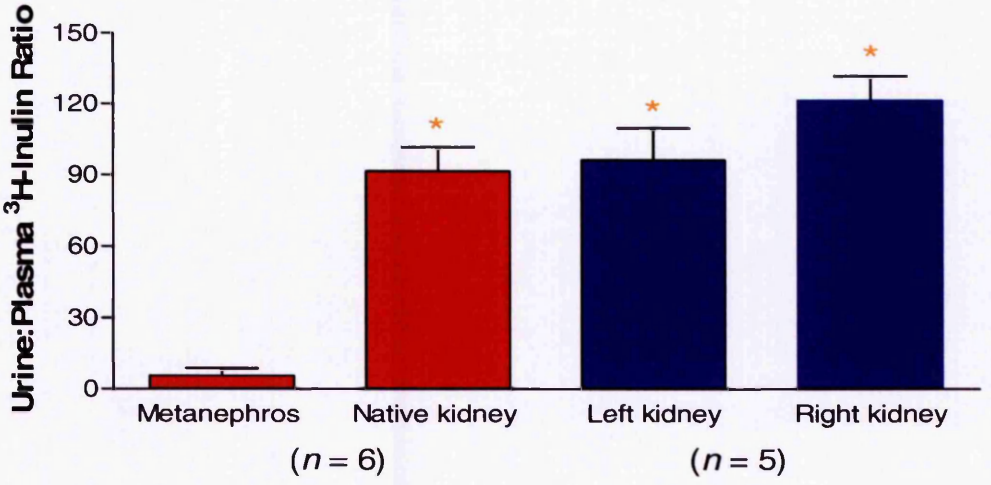

Figure 2.17. Urine:plasma ^{3}H-inulin ratios in transplanted metanephroi and controls. Urine:plasma ratios of ^{3}H-inulin measured in transplanted metanephroi and remaining right native kidney of animals with transplants (red bars), and left and right kidneys of control animals (blue bars). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA, following $\log_{10}$ transformation of data, and Tukeys post-hoc test. $P < 0.05$ was deemed significant. * $P = 0.001$ compared to metanephroi.

It was intended that the electrolyte concentrations of urine from the metanephros would also be measured. However, the volume of urine collected from the metanephroi at the end of the three-hour clearance period was very small (around 20-25 µl). Consequently, after taking

samples for analysis of ^{3}H-inulin and PAH, there was insufficient urine left to analyse the electrolyte concentrations.

2.3.2.6 Urea concentrations

Urea concentrations (figure 2.18) were significantly lower in serum from animals with transplanted metanephroi compared with cyst fluid and urine from both the metanephros and native kidney (all P = 0.001). The concentration of urea in cyst fluid was significantly lower than that of both urine produced by the metanephros (P = 0.004) and urine from the host's remaining native kidney (P = 0.001). The concentration of urea in urine from the metanephros was significantly lower than that of urine produced by the native kidney (P = 0.001).

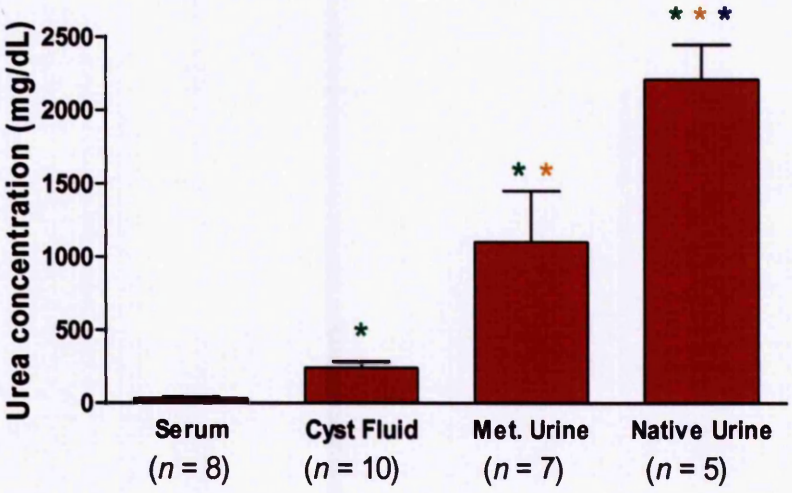

Figure 2.18. Urea concentrations in transplanted metanephroi and controls. Urea concentrations of serum from animals with transplanted metanephroi, fluid-filled cysts observed around the ureter of transplants at 3 weeks post-transplant (Cyst fluid), urine produced by the transplanted metanephroi following a 3-hour collection period of the clearance experiment (Met. Urine) and urine produced by the one remaining native kidney following a 15 minute collection period of the clearance experiment (Native Urine). All data are presented as mean ± S.E.M. Statistical analysis was by one-way ANOVA. $P < 0.05$ was deemed significant. * $P = 0.001$ compared to serum, * $P = 0.004$ (met.urine) and $P = 0.001$ (native urine) compared to cyst fluid, $^{\bullet}P < 0.001$ compared to met urine.

2.4 Discussion

2.4.1 Pilot in vivo *study*

2.4.1.1 General observations

In contrast to commonly used laboratory rat strains, such as Sprague-Dawley and Wistar rats, there are very few reports of *in vivo* renal function in the Lewis rat. This strain was chosen for the current study because it is highly inbred, hence, when tissue is transplanted from one rat to another there is a very low risk of rejection. This is largely due to the fact that the major-histocompatibility (MHC) status of the rats is the same and transplanted tissue is not recognised as 'foreign' by the host's immune system, allowing the transplant to grow without risk of graft rejection and necrosis. Consequently, it was important to establish baseline values for a range of renal variables before comparisons between transplanted metanephroi and native, host tissue could be made. Furthermore, it was essential to establish the most appropriate combination of surgical access and infusion regime in order to maximise the data that could be collected from transplanted metanephroi.

Before considering the measured renal variables in detail, it is useful to comment on the overall trends observed during the generation of baseline measurements for the Lewis rat. Clearly, animals that had an indwelling bladder catheter had lower filtration and excretion rates than those with both ureters cannulated. Likewise, animals undergoing the SERVO clearance protocol tended to have lower outputs than their constant infusion rate counterparts.

In the case of bladder vs ureters, the results, at first glance, appear unexpected. In order to cannulate both ureters a laparotomy is required. This is likely to cause greater physiological stress upon the animal and blood loss, however small, is more likely during this procedure. In contrast, inserting a bladder catheter is very simple and quick, with only a small incision required. Despite the apparent increased risk of blood loss and stress during ureter cannulation, there were no obvious identifiers that this had occurred. Mean arterial pressure (MAP), throughout both the equilibration period and experimental phase of the study, was stable and comparable between groups. MAP was also comparable with other groups that have studied anaesthetised Lewis rats and reported MAPs in the range of 100-110 mm Hg (Kunes et al., 2002; Schumacher et al., 2002).

It is widely accepted that cannulating the bladder leads to a problem with fluid accumulating within the bladder to form a 'pooling effect' known as 'dead space', (Malvin and Fusco, 1967; Fejes-Toth et al., 1983). However, one would assume that during the 6-hour clearance protocol, any effect due to bladder 'dead space' would be minimal as long as flow rates are sufficiently high to force the urine down the catheter. Certainly, bladder dead space would not be sufficient to cause the differences observed.

The ureters are composed of smooth muscle and transport urine from the kidneys to the bladder via a series of peristaltic movements (Calafati and Zanaboni, 1954; Zanaboni et al., 1954). These peristaltic actions could act to 'siphon' urine down the ureter catheter and so be more representative of urine flow, particularly at low flow rates. The bladder, on the other hand, despite also being composed of smooth muscle, does not undergo

peristalsis. The urinary bladder, being a reservoir, does not usually give rise to a constant flow of urine but is normally emptied at periodic intervals. Emptying of the bladder in these clearance experiments was reliant on gravity, with the collecting reservoirs sitting below the level of the anaesthetised animal. This difference in action, either forced contractions (ureter) or passive flow (bladder) may help to explain the observed difference in flow rates. As a consequence of lower flow rates, when applied to the clearance equations, the calculated values for GFR, ERBF, osmolar and electrolyte excretion rates would also be reduced. This could mean that these values may not be a true indication of the actual output by the kidney.

Comparing the effect of SERVO infusion versus a constant infusion rate on measured renal variables, the reason for the general trend towards lower values observed in the former group may be quite simple. Those animals receiving a constant infusion of 50 $\mu l\ min^{-1}$ underwent a gradual increase in urine flow rate during the equilibrium period. Full diuresis was not achieved until 2-3 hours into the experiment. This was likely to lead to an increase in extracellular fluid (ECF) as a result of 'water-overloading'. Following this increase in ECF, vasopressin (AVP) secretion would decrease, leading to a reduction in water reabsorption in the collecting ducts and ultimately an increase in water excretion, until a normal ECF volume was reached (Ferguson, 1954; Andersson et al., 1982). This increase in urine flow rate was often observed towards the end of the equilibrium period. Throughout the experimental 3-hour phase of the clearance study, animals on a constant infusion with ureters cannulated

tended to excrete urine at a rate comparable with the volume of dextrose infused, with flow rates of between 40-60 μl min^{-1} g kid wt^{-1}. Bladder cannulated animals did not achieve this level of diuresis, with rates of between 15-20 μl min^{-1} g kid wt^{-1} throughout the experimental period, reiterating the influence of cannulation type on the measured outcome. SERVO infused animals, on the other hand, did not receive such large volumes, as the infusion rate was matched to spontaneous urine output. As a result, ECF was not expanded and the rats did not undergo such a marked diuresis, hence, lower excretion rates were recorded.

2.4.1.2 Effective renal blood flow

The most common constant infusion protocol used to measure ERBF involves the use of a single bladder cannulation. Values obtained using this approach fell within the range 2-6 ml min^{-1} g kid wt^{-1}. The value of 3 ml min^{-1} g kid wt^{-1} obtained herein is at the lower end of the range but, nonetheless, is consistent with reported data (Haylor et al., 1996; Ferreira et al., 2005; Ovcharenko et al., 2006).

All experiments in this pilot study, and later in the transplantation study, were carried out on female rats. In contrast, most published literature describes renal function in male rats. There are gender differences in a number of renal variables: for example female rats secrete PAH at significantly lower rates than male rats (Cerrutti et al., 2001) though this would not affect the measured ERPF. Nonetheless, ERBF rates measured in this study were comparable with previously reported values obtained in

male Sprague-Dawley rats undergoing the same protocol in this laboratory (Bogzil et al., 2005).

There are no published data on rats with cannulated ureters undergoing the SERVO control infusion technique. Nonetheless the blood flows measured in the SERVO-controlled group with ureter cannulations appear to fall within the normal, reported range for renal blood flows of between 2-6.5 ml min^{-1} g kid wt^{-1} in the literature (Walker et al., 1983; Haylor et al., 1996; Martins et al., 2003; Koura et al., 2004; Bogzil et al., 2005; Ferreira et al., 2005). The only report in which RBF was measured in a constant infusion model with a double ureter cannulation gave a value of around 4.4 ml min^{-1} g kid wt^{-1} (Hammad et al., 2000). This is almost identical to the value of 4.38 ml min^{-1} g kid wt^{-1} in the current experiment.

The differences in ERBF measured in the various cannulation and infusion groups do not appear to be due to any changes in systemic blood pressure, as MAP was comparable for all groups. This suggests that cardiac output and the volume of blood delivered to the kidneys each minute would have been fairly consistent across the groups. Thus, there must have been other reasons for the observed differences in renal blood flow between groups. As outlined in the methods section 2.2.4.3, urine flow rate is required in order to calculate ERBF. If the estimate of urine flow rate, particularly in the bladder-cannulated group, were not entirely representative of true UV, then the calculated blood flow will be lower than the true flow rate. There is further evidence to support this hypothesis from the raw PAH values. The PAH concentrations in urine were fairly consistent across the groups, as was plasma PAH concentration, implying

that the intrarenal urine flow rates were comparable between groups. This suggests that the differential factor may indeed be the collection of urine and hence the (under) estimation of urine flow rate. If this is indeed the case, this under estimate of urine flow rate will have implications for all other variables calculated on the basis of this term.

2.4.1.3 Glomerular filtration rate

Glomerular filtration rate measurements in SERVO control animals with bladder cannulations can be compared with several previous studies undertaken in this laboratory (Ahmed et al., 2003; Bogzil et al., 2005; Rad et al., 2005). The previously reported GFR data range from 0.5-1 ml min^{-1} g kid wt^{-1}, comparable with the data from the current experiment. The GFRs of bladder-cannulated controls on a constant infusion rate are also comparable with those of others, ranging from 0.8-1.5 ml min^{-1} g kid wt^{-1} (Walker et al., 1983; Gouldsborough and Ashton, 2001; Sahajpal and Ashton, 2003; Song et al., 2006).

There are some limited data on GFR in animals undergoing infusion of 0.9% saline at a constant rate of 50 µl/min with both ureters cannulated (Dragun et al., 1998; Hammad et al., 2000). These groups reported GFRs of 0.7-0.9 ml min^{-1} g kid wt^{-1}, which are much lower than those in the current experiment. However, there are numerous differences between these protocols, including the strain and sex of rats used. Thus, any comparison should be treated with caution. Also, in the study by Dragun *et al* (1998) only 1 hour was allowed for equilibration, compared with three

hours in the current experiments. Experience in this laboratory suggests that urine flow rates do not approach the constant infusion rate until 2-3 hours have elapsed. Hence shorter equilibration periods may result in an under estimation of GFR.

The likely reasons for the observed differences in GFR in bladder-cannulated animals and animals on the SERVO system are the same as those outlined for renal blood flow, namely an under estimation of the true urine flow rate. However, if the blood flow values are indeed representative of what is occurring within the kidney, then a reduced blood flow means that there will be a reduction in the hydrostatic pressure within the glomerular capillaries. This will result in a reduction in the force favouring filtration and so, in the absence of compensatory changes in glomerular arteriole diameter, GFR will fall. The reason for this underlying reduction in RBF is unclear.

2.4.1.4 Urine flow rate

Values obtained for UV were generally comparable with previously published data. Animals infused via the SERVO-controlled system with bladder cannulations had UVs comparable with others at around 10 µl min^{-1} g kid wt^{-1} (Bogzil et al., 2005; Rad et al., 2005), whereas those with ureter cannulations on a constant infusion had higher UVs compared with the published data of 12.5 µl min^{-1} g kid wt^{-1} (Hammad et al., 2000). This, again, is probably due to differences in the clearance protocols employed, as described above. Animals on a constant infusion and with a single bladder catheter again showed urine flow rates comparable with published

data in the range of 10-30 µl min^{-1} g kid wt^{-1} (Walker et al., 1983; Gouldsborough and Ashton, 2001; Beltowski et al., 2004; Song et al., 2006).

In the SERVO experiments, infusion rates tended to be lower than was the case in the constant infusion groups, as one might expect. The infusion rate in the SERVO experiment is driven by spontaneous urine output (10-20 µl/ min), which is lower than the constant infusion rate of 50 µl/min used herein. Cannulation type had a significant effect on UV, with the flow rate of the UC group being around twice that of the US group. This was also the case when comparing BC to BS groups. It may be the case that urine was pooling in the bladder rather than flowing down the catheter immediately and thus flow rates were not entirely accurate. If this is the case results have to be interpreted with a degree of caution, as most of the parameters measured are calculated by using the urine flow rate. A reduced flow rate would lead to a subsequent decrease in the calculated ERBF, GFR and excretion rates of electrolytes and other solutes. Despite this, however, the UV results gained in the current study were no different from the range of published data, in keeping with normal values for animals under these conditions.

2.4.1.5 Osmolar excretion and clearance rates

There were some statistical differences between groups for plasma osmolality (P_{osm}), with the BC group and the ureter-cannulated controls having statistically lower P_{osm} than the BS group. The P_{osm} of the BS group and the BC group however, at around 270-290 mOsm kg^{-1} compared favourably with previously published literature (Sebaai et al., 2002; Salas et al., 2004; Bogzil et al., 2005; Guron, 2005). The P_{osm} for the UC and US groups however, at around 250 mOsm kg^{-1}, appear slightly low. This is likely to be because of the low plasma sodium concentrations also seen in these ureter-cannulated animals, which would lead to a diuresis, hence the higher urine flow rates observed in the UC and US group. Despite these differences observed in P_{osm}, they were not sufficient to cause a change in the haematocrit, which was normal in all groups at around 40-44 %.

A review of the literature failed to identify any studies in which osmolar excretion or clearances were described in rats with ureter cannulations. There are more data for bladder-cannulated animals, however results were difficult to compare as most groups used infusion rates twice that of the rate used in this study (Blandford and Smyth, 1988; Smyth et al., 1988) or carried out these studies on conscious rats (Haylor and Lote, 1980; Bliss and Lote, 1982).

The observed differences in osmolar excretion and clearance appear to show the same trend as those for urine flow rate. This is not surprising since lower flow rates mean that water is being reabsorbed, largely via active reabsorption of sodium.

2.4.1.6 *Renal handling of sodium*

Sodium excretion, clearance and fractional excretion tended to be lower across all groups when compared with previously published data of around 0.7-1.5 µmol min^{-1} g kid wt^{-1} for $U_{Na}V$, 5-12 µl min^{-1} g kid wt^{-1} for C_{Na} and 0.9-1.7 % for FE_{Na}. (Hammad et al., 2000; Bogzil et al., 2005; Guron, 2005; Rad et al., 2005; Song et al., 2006). The most obvious explanation for this is that 2.5% dextrose was infused in the current study rather than 0.9% saline, which was used in most other studies. Saline was considered to be an inappropriate vehicle in the subsequent experiments with transplanted metanephroi. Due to their much reduced size and potential lack of development, metanephroi may not able to excrete sodium appropriately. This would lead to salt-overload of the transplant and potential distortion of the limited functional capacity. Consequently, dextrose was chosen as the vehicle for infusion. This has the advantage of an osmotic diuresis effect, if immature tubules cannot cope with the filtered load of glucose and so water is lost along with glucose in the urine.

The results showed that cannulation type and choice of infusion protocol were both deciding factors in the differences observed. As sodium excretion and clearance are not dependent on flow rate the reasons for these differences are largely unknown.

2.4.1.7 *Renal handling of potassium*

Like sodium, potassium excretion and clearance rates, together with fractional excretion tended to be slightly lower in the current experiments than other published results. These published data were in the range of 0.8-2 µmol min^{-1} g kid wt^{-1} for U_KV, 240-360 µl min^{-1} g kid wt^{-1} for C_K and 40-50 % for FE_K (Hammad et al., 2000; Walter et al., 2000; O'Flaherty et al., 2001; Sahajpal and Ashton, 2003; Bogzil et al., 2005; Rad et al., 2005; Song et al., 2006). The observed differences in potassium handling are again likely to be linked to a perceived artefactual decrease in UV in the bladder-cannulated group and thus the increased U_KV and C_K values in the UC group.

P_K across all groups was consistent and around normal levels of 3.5-4.5 mmol/L previously seen in the literature (Walter et al., 2000; Bogzil et al., 2005; Guron, 2005).

2.4.1.8 *Conclusions of pilot experiments*

The pilot study fulfilled its two major aims: to generate a set of baseline physiological measurements for female Lewis rats, and to compare infusion and cannulation techniques. Values obtained were generally comparable with published data, indicating that experimental techniques were repeatable and gave reliable data. On the basis of these observations, it was decided that, given the reported low flow rates in metanephroi (Rogers et al., 1998; Rogers and Hammerman, 2001a) the

SERVO infusion protocol was not likely to result in the production of adequate volumes of urine for analysis.

As the constant infusion protocol resulted in higher GFR measurements and urine flow by comparison with the SERVO infusion approach, this was chosen as the method for infusion in the subsequent experiments. A slightly lower infusion rate of 40 $\mu l\ min^{-1}$ was chosen, taking into account that only one functioning native kidney remained in the host animals and so excretory capacity was diminished.

On the basis of the results for the bladder vs ureter analysis, the best approach seemed to be to cannulate both ureters in the subsequent experiments in host rats. However, this carried the added risk of damage to the ureter anastomosed to the metanephros' ureter. So, in order to minimise damage to the anastomosed ureter and the risk of leakage from the connection site, metanephric urine was collected from the bladder. Dilution with urine from the contralateral kidney prior to the experiment was minimised by compressing the bladder to expel all urine before inserting the collection cannula. The bladder was compressed again at the end of the experiment to ensure that all metanephric urine was collected. Urine produced by the host's remaining kidney was collected via a separate ureter cannula.

2.4.2 Animals with transplanted metanephroi

2.4.2.1 Measure of stability of animals during clearance

Table 2.2 indicates that animals with transplanted metanephroi were in a physiologically stable state during the clearance experiments. Mean arterial pressure was stable throughout the 6-hour clearance experiment and was comparable with control animals. The haematocrit values obtained for animals with transplanted metanephroi were also comparable with control animals. It is widely accepted that mammals possessing a single kidney are able to function normally with no obvious deterioration in renal function or overall health of the animal, provided the reduction in renal mass occurs in adulthood. If this loss of renal mass occurs in neonates, there is subsequent hypertension in adulthood and a deterioration in renal function (Woods, 1999; Moritz et al., 2002). It has been shown in human adult kidneys that the loss of up to 70% of nephrons does not appear to affect the ability of the kidney to excrete water and solutes appropriately. This is thought to be achieved through a compensatory mechanism by the remaining nephrons (Guyton and Hall, 2000).

In the current study there appeared to be some compensatory growth of the remaining native kidney in the unilaterally nephrectomised animals compared with controls. This is apparent in both the 'wet' kidney weight and kidney weight expressed as a percentage of body weight. This increase in renal mass is likely to be as a direct result of hypertrophy, as

has been described previously in animals following unilateral nephrectomy (Mason and Ewald, 1965; Anderson, 1967; Ghose et al., 1970).

Perhaps the more striking point to note in table 2.2 is the weight of the transplanted metanephroi. These transplants were explanted following the clearance study, which was around 3-4 months post-transplant. A normal kidney developing *in situ* for 3-4 months would be expected to be the same size as the kidneys harvested from control adult rats. However, the transplanted metanephroi were only around 6% of the weight of the mean control left kidney. The possible reasons for this stunted growth will be discussed in detail both later in this chapter and in Chapter 3.

2.4.2.2 Effective renal blood flow and renal vascular resistance

The effective renal blood flow (ERBF) reported herein is the first estimation of blood flow through transplanted metanephroi. As such, this represents an important advance in the characterisation of the model. Clearly, renal blood flow through the transplants is much lower than that through the one remaining native kidney of the host animal and kidneys of control animals. Even when standardised for the renal mass present, ERBF in the metanephroi remains very low. This contrasts with previous reports on developing kidneys which have shown that effective renal blood flows, per gram of renal tissue, between 3-4 weeks of age (Guron, 2005) and 12 weeks of age (Sanders et al., 2005) are comparable with values expected in a normal adult rat. The transplanted metanephroi in this study, at time of clearance, were around 16 weeks of age. Clearly, they do not display a level of renal blood flow consistent with this chronological age. However,

the figure quoted is an estimate of effective renal blood flow. In order to give a more accurate renal blood flow measurement, the PAH concentration within the venous outflow of the transplanted metanephros would have to be measured. Unfortunately, it is very difficult to locate the metanephric renal vein and so collection of venous blood was not possible. It is also known that the tubules of neonatal rats secrete PAH to a lesser extent compared with adult kidney tubules (Horster and Lewy, 1970; Braunlich, 1985). Thus, this effective renal blood flow measurement may be an under-estimate. However, filtration fraction, described later, was normal in transplanted metanephroi suggesting that the measurement of renal blood flow was fairly accurate.

It is widely accepted that deep anaesthesia contributes to a decreased renal blood flow (Walker et al., 1983) but this does not explain the marked difference observed between transplanted metanephroi and the remaining adult native kidney. One reason for this decrease in ERBF is likely to be the marked increase in renal vascular resistance observed in transplanted metanephroi. In normal mammalian kidneys, an increase in renal vascular resistance is usually a result of vasoconstriction in vessels upstream of the glomerulus, such as the afferent arteriole (Gattone et al., 1983), usually as a compensatory response to an increase in systemic blood pressure. In the current experiment, blood pressure in animals with transplanted metanephroi was not different from that in control animals, so it is unlikely that the high RVR of the metanephroi was due to compensatory vasoconstriction of afferent arterioles. However, given that the size of the metanephroi is similar to a neonatal kidney and that MAP in a developing

embryo is much lower than that in an adult (Gray, 1984; Chevalier et al., 1996), it is possible that the metanephroi were responding to a perceived increase in blood pressure. However, a more likely explanation for the large vascular resistance observed comes from observation of the vascularisation of the transplants. Recent work has shown that vascularisation of transplanted metanephroi is primarily as a result of angiogenesis from the host rather than vasculogenesis from the transplant itself (Takeda et al., 2006). However, this does not appear to proceed normally as, rather than developing a distinct, single renal artery, a number of smaller arteriolar type vessels develop (Hammerman, 2002). This, coupled with a lack of arteriogenesis, means that the vascular tree consists of a series of small, high resistance vessels. Consequently, it is perhaps not surprising that RVR is elevated and blood flow is reduced.

The other interesting feature to note was that the remaining native kidney of animals with transplants had a significantly increased blood flow compared with the left kidney of control animals. This was associated with an overall increase in the mass of the native kidney. Renal plasma flow has been shown to increase in the contralateral kidney following unilateral nephrectomy in a number of studies (Northrup and Malvin, 1976; Sachtjen et al., 1977; Lopez-Novoa et al., 1982). This appears to represent a compensatory mechanism for the loss of total nephron number.

2.4.2.3 Glomerular filtration rate

The low GFR of the transplanted metanephroi, compared with the native kidney and control animals, can be attributed to one major reason: reduced renal blood flow. This reduction in renal blood flow, would, in turn, result in a reduction in hydrostatic pressure in the glomerular capillaries and so a low GFR. Systemic blood pressure during the clearance procedure was constant and within a normal range, meaning that any alteration in glomerular hydrostatic pressure was not likely to be as a result of any reduction in mean arterial pressure.

However, there may be a simpler reason why GFR and other parameters were reduced in the transplanted metanephroi. Weights of the metanephroi, up to 4 months post-transplant, were only comparable with kidneys of rats at around time of birth (E21 to PND1 rats) (M.Dilworth and N.Ashton, unpublished observations). Hence, it is logical to suggest that if these transplants do not develop at a normal rate, their physiological performance may also be limited. This is supported by published data on GFR in neonatal rats as early as postnatal day 4 (Gray and Kavlock, 1991). This GFR value of around 0.09 ml min^{-1} g kid wt^{-1} is fairly close to the GFR obtained from the transplanted metanephroi in the current study. Considering that the transplanted metanephroi are similar in size to a normal postnatal day 0 rat kidney, the evidence suggests that the transplants are performing as would be expected for their size and stage of development.

In the normal animal, there is a period of rapid growth and development, both of renal mass and filtration rate, over the postnatal period. The postnatal day 4 GFR increases from 0.09 ml min^{-1} g kidney wt^{-1} (Gray and Kavlock, 1991) to 0.35-0.46 ml min^{-1} g kidney wt^{-1} by postnatal day 10-17 (Fleck, 1992; Chevalier et al., 1996). Indeed, GFR in the rat does not approach that of the adult, per gram of kidney tissue, until 3-4 weeks of age (Guron, 2005).

It is important to remember that standardising the results by kidney weight does not take into account glomerular, and thus nephron, number. An estimate of glomerular number in the transplanted metanephroi is required to determine whether the number of nephrons present is appropriate to the mass of tissue. If the nephrons are not tightly packed or are interspersed with connective tissue, standardising the data per gram of kidney tissue may not allow direct comparisons across different ages/groups. An assessment of nephron number would also be indicative of the potential maximal functional capacity that the metanephros may achieve.

A key finding during these clearances was that, despite the lower GFR of the transplants, filtration fraction appeared normal at approximately 0.21. Thus, around 20% of the blood flow through the transplant was being filtered, a figure comparable with the native kidney in this study (27 %) and adult functioning kidneys in other studies (Walker et al., 1983; Vos et al., 2001; Ashton et al., 2003). This strongly suggests that blood supply to the metanephros is a crucial limiting factor in the physiological performance of the transplant. Since filtration fraction appears normal, it begs the

question, would an increase in blood flow to the metanephros result in an increase in GFR?

GFR in the host's remaining native kidney was comparable with both kidneys of the control animals. Given the compensatory increase in renal blood flow of the native kidney, it appears reasonable to assume that GFR would also show some form of compensatory response, as seen in previous papers following unilateral nephrectomy (Diezi et al., 1976; Provoost and Molenaar, 1980; Lopez-Novoa et al., 1982; Haylor et al., 1996). However, despite the compensatory hypertrophy exhibited by the one remaining native kidney, a subsequent increase in GFR failed to occur. The reason for this is unclear, but lies beyond the scope of the present study.

2.4.2.4 Urine flow rate

Although gross flow rates of urine from the metanephros were very low, when standardised to kidney weight they were not statistically different from control tissue. This may be a statistical anomaly, in part due to the large standard error for the native kidney. However, these data show that, per gram of renal tissue, transplanted metanephroi are capable of producing urine at a rate approaching that of adult kidneys. The mean UV of 3 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$ is comparable with previously published flow rates by transplanted metanephroi in rats (Rogers et al., 1998; Rogers and Hammerman, 2001a).

If GFR in transplanted metanephroi is converted to $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$, the mean is 17 $\mu l\ min^{-1}\ g\ kid\ wt^{-1}$: clearly this amount of fluid was not excreted, suggesting that tubular reabsorption of water has occurred. This implies that the transplanted metanephroi have some capacity to reabsorb water. There is further evidence for this from the urine:plasma inulin ratios. Inulin counts in metanephric urine were around 3-4 times higher than those found in plasma, suggesting that there had been some degree of water reabsorption. Clearly this is not as well developed as the native and control adult kidneys, in which the urinary inulin counts were around 80-100 times greater than those of plasma. Unfortunately, due to the extremely low volume of urine collected from each metanephros, typically around 20-25 µl, concentrations of electrolytes and the osmolality of the metanephric urine could not be measured. These measurements would have allowed a far greater understanding of the concentrating ability of the transplants *in vivo*, as well as providing an idea of how well developed the tubules of the metanephroi were.

There is some evidence to support the suggestion that transplanted metanephroi have the ability to reabsorb water and partially 'concentrate' urine. Previous work carried out by our group showed that the osmolality of urine collected from animals surviving on metanephros transplants alone (for around 48-72 hours longer than animals with no renal tissue whatsoever) was in the region of 400-500 mOsm/kg (Marshall et al., 2006 *In press*) compared with typical values in normally hydrated rats of 800-1200 mOsm/kg (Horst et al., 1988; Bonilla-Felix and Jiang, 1997; Guron, 2005; Rojek et al., 2006). These observations suggested that the

metanephroi were only able to 'concentrate' urine to modest levels, much like neonatal rats (Liu et al., 2001; Kim et al., 2002). However, this value of around 400-500 mOsm/kg is only slightly below published levels in the urine of rats 4 days after birth (around 590 mOsm/kg) (Gray and Kavlock, 1991) implying a more mature 'concentrating' mechanism than its size would suggest. In fact, it has been shown that rats are only able to concentrate urine to adult levels by 3-4 weeks of age (Guron, 2005). Clearly, transplanted metanephroi are not as mature as a 3 or 4 week old kidney and so should not be expected to 'concentrate' urine to the same degree.

The sodium concentrations in the urine produced by the animals surviving on the transplanted metanephroi alone were elevated compared with controls, whilst potassium concentrations were much lower. This possible reduced ability to reabsorb sodium and secrete potassium may be a further indicator of tubular immaturity (Marshall et al., 2006 *In press*).

UV for both animals with transplants and control animals was less than the infusion rate of 40 μl/min, suggesting that fluid retention occurred, even though haematocrit was normal, leading to an increase in extracellular fluid volume (ECFV). This excess fluid appeared to pool in the peritoneal cavity of most animals. Upon laparotomy at the end of the experiment, the peritoneal space was fluid-filled. However, haematocrit was within the normal range, which explains why ECF did not cause the production of an osmotically dilute urine, as would be expected.

2.4.2.5 *Urea concentrations*

Concentrations of urea in plasma, cyst fluid and urine produced by the metanephros and native kidney are comparable with previous reports in rat (Rogers et al., 1998), human and porcine transplants (Dekel et al., 2003). The trend observed is interesting, since it appears to reflect renal development. For example, cyst fluid was taken at around 3 weeks after initial transplantation and its urea content was significantly lower than that in urine from the metanephros following clearance, at around 4-5 months after transplantation. This strongly suggests that between 3 weeks and 3 months following metanephros transplantation, the transplant develops a greater concentrating ability. This is likely to be due to tubular development. Likewise, urine from the native kidney had around twice the urea concentration of urine from the metanephros following clearance. This is evidence that the transplants' ability to concentrate urine, in terms of urea, is still relatively limited. However, by again comparing transplanted metanephroi with kidneys from 4-day old animals (Gray and Kavlock, 1991) it is apparent that the transplants have a greater concentrating ability than their mass might suggest. Urea concentration in metanephric urine was around 30 times that of serum, compared with around a 20-fold difference between urine and serum in 4-day old animals. Therefore, relative to size, the transplants appear to be more mature than would be expected. This proposal that transplanted metanephroi have an immature 'concentrating ability' may be further supported by the urine:plasma inulin ratios. Although the transplants may be showing evidence of water reabsorption to 'concentrate' inulin in the urine to around 3-4 times the

concentration found in serum, this is much less than the urea ratio, and well below the 80-100 fold difference observed in inulin handling by the adult control kidneys.

Not much is currently known about the ability of the transplanted metanephroi to concentrate urine, particularly in terms of the presence of the key transporters and channels. These include the urea transporters, aquaporins and sodium channels (Fushimi et al., 1993; Promeneur et al., 1996; Fernandez-Llama et al., 1998; Li et al., 2001; Tian et al., 2006).

Finally, it is worth noting that, despite the reduced ability of the transplant to excrete urea in the urine, plasma urea concentrations were around normal physiological levels. This provides further evidence that the one remaining native kidney was able to maintain body homeostasis without the need for a second functioning kidney.

2.4.2.6 Conclusions for animals with transplanted metanephroi

This study is the first to report renal blood flow and renal vascular resistance in transplanted metanephroi. Together with estimates of GFR of a similar magnitude to those reported previously (Rogers et al., 1998; Dekel et al., 2003), these data show that transplanted metanephroi function at the lower end of the physiological range for their size. Clearly, both GFR and ERBF will have to be improved in order to increase flow rates and produce viable transplants that can sustain life. This is not an unrealistic goal, as it has already been shown that rats survived with no native renal tissue on a transplanted metanephros alone up to 3 days longer than rats with no renal tissue whatsoever (Rogers, 2004).

Furthermore, in this study rats only received a single functioning transplant. In larger species, with more space to perform multiple ureteroureterostomies there is no reason why multiple transplants could not be connected to give a cumulative function. Indeed, a preliminary study in our laboratory has shown that the transplantation and subsequent connection of two metanephroi in the rat further extended life by up to a day compared with rats receiving a single transplant (Marshall et al., 2006 *In press*). While the option of using multiple transplants offers hope for successful prolongation of life in the future, the greatest improvements will come from promoting growth and maturation of the individual metanephros. If an improved blood flow could be delivered, this may well improve GFR and thus the excretory performance of the transplants. As well as improved blood supply and flow, tubular maturity of the transplants also needs to be improved. This would lead to a greater ability to concentrate urine. It may be the case that improved blood supply to the transplant also improves growth of the transplant and the tubules mature as a result. Despite these current limitations, this work adds further knowledge to the area of metanephros transplantation and identifies areas that need to be improved in order for this work to be taken forward towards a clinical setting. The next chapter will focus on the assessment and quantification of tubular maturity in transplanted metanephroi.

Chapter 3: Assessment of glomerular number and tubular maturity of transplanted metanephroi

3.1 Introduction

3.1.1 Background

As described in Chapter 2, transplanted metanephroi between 3 weeks to 4 months post-transplantation are, in terms of weight, comparable with rat kidneys at around the time of birth. However, the number of functioning nephrons within the transplants remains unknown. An estimate of nephron number would help to determine if the transplants are currently functioning at normal levels for their size, or if there is scope to improve the function by increasing nephrogenesis. The second unknown feature is the maturity of the tubules in the transplanted metanephroi. Data presented in Chapter 2 suggest that the tubules were relatively immature, specifically in terms of the transplant's ability to concentrate inulin and urea. Thus, a study to examine the transporters, channels and receptors involved in the concentration of urine is required. These targets include the urea transporters A-1,2 and 3 (UT-A1, 2 and 3), aquaporins 1 and 2 (AQP1 and 2), the epithelial sodium channel (ENaC) and the Na-K-2Cl co-transporter type 2 (NKCC2). A schematic showing the localisation of these channels and transporters can be seen in figure 3.1.

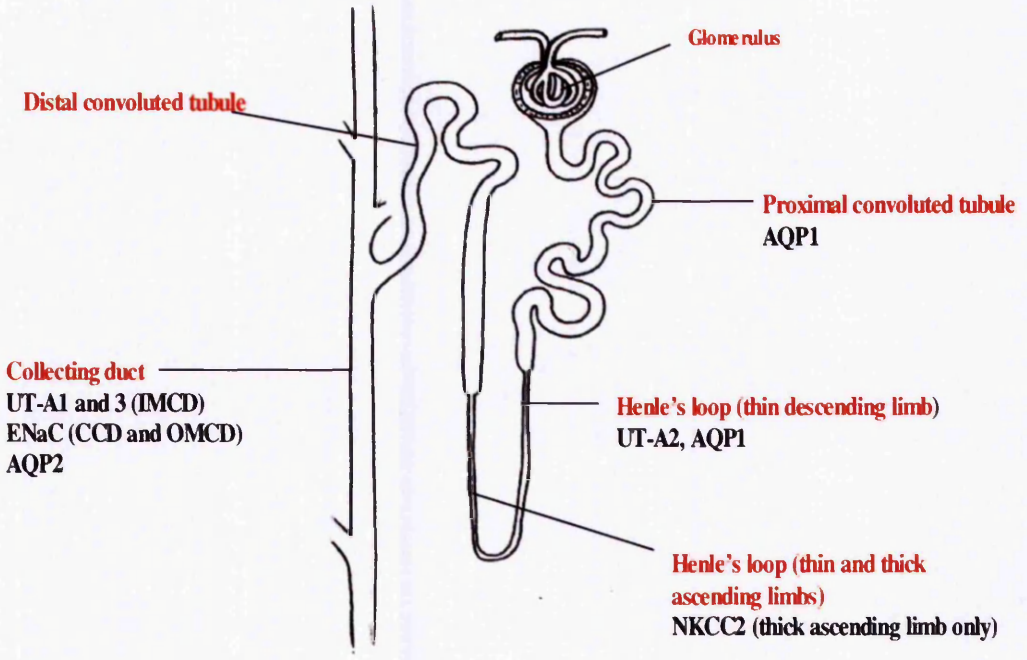

Figure 3.1. Nephron schematic. Showing the localisation of channels and transporters that are key to the urinary concentrating process. IMCD, inner medullary collecting duct; CCD, cortical collecting duct; OMCD, outer medullary collecting duct

Urea transport is crucial in the formation of a concentrated urine. Urea has a low lipid solubility and if specific urea transporters are not present, it crosses cell membranes by passive diffusion. Movement of urea within the inner medullary collecting duct (IMCD) and descending thin limb of Henle's loop, normally, is via specific urea transporters located in the tubular cell membrane (Sands et al., 1997). It is these transporters, named the urea transporters, which are able to facilitate the movement of urea against a concentration gradient. This movement, particularly within the IMCD aids the final concentration of urine.

UT-A1 and UT-A3 have been localised in the kidney within the inner medullary collecting duct (Nielsen et al., 1996; Shayakul et al., 1997; Shayakul et al., 2001; Terris et al., 2001) whilst UT-A2 is expressed in the

thin descending limbs in both the inner and outer medulla (Shayakul et al., 1997; Wade et al., 2000). UT-A1,2 and 3 have all been shown to be crucial to urea transport and the formation of a concentrated urine (Sands et al., 1997; Fenton et al., 2004).

It is not only urea transport that is responsible for the concentration of urine. The final concentration of urine is dependent on the osmotic gradient within the medulla and the water permeability of the collecting ducts. AQP1 plays a crucial role in the re-absorption of water, particularly in the formation of the medullary osmotic gradient by the loop of Henle' and is expressed within the kidney in the proximal tubule, descending thin limb of Henle's loop and descending vasa recta (Sabolic et al., 1992; Nielsen et al., 1993; Nielsen et al., 1995). AQP1 plays a crucial role within the proximal tubule where the bulk of glomerular filtrate is re-absorbed. The presence of AQP1 within the vasa recta and thin descending limb (tDL) is thought to be important in the establishment of the medulla's hypertonic environment, largely by the re-absorption of water (Ma et al., 1998).

NKCC2 has been localised to the thick ascending limb (TAL) and the macula densa (Ecelbarger et al., 1996; Kaplan et al., 1996; Obermuller et al., 1996; Nielsen et al., 1998) and is also vitally important in the formation of a concentrated urine (Simon et al., 1996). NKCC2 is the major pathway for salt transportation within the apical membrane of the TAL and, via salt reabsorption, is crucial in the production and maintenance of countercurrent multiplication.

AQP2 is thought to be the most crucial aquaporin in the kidney in terms of its role in the concentration process. AQP2 in the kidney is expressed solely in the principal cells of the collecting duct (Fushimi et al., 1993) and plays a major role in the concentration of urine (Deen et al., 1994; Rojek et al., 2006). Vasopressin (AVP) increases water permeability within the collecting duct (Grantham and Burg, 1966; Morgan et al., 1968) by increasing AQP2 expression (Fushimi et al., 1993; Knepper, 1997). AVP stimulates the shuttling of AQP2 protein from vesicles to the apical plasma membrane, an idea first proposed by Stetson *et al* (Stetson et al., 1980). Thus, an increase in vasopressin leads to an increase in water permeability and thus water re-absorption within the collecting duct, largely via the insertion of AQP2 into the apical plasma membrane.

ENaC is composed of 3 homologous subunits, α, β and γ (Canessa et al., 1993; Lingueglia et al., 1993; Canessa et al., 1994; McDonald et al., 1994; McDonald et al., 1995) and is expressed on the apical membranes of kidney cortical collecting duct (CCD) and outer medullary collecting duct (OMCD) cells (Tousson et al., 1989). ENaC plays a key role in salt and water reabsorption, largely by the actions of aldosterone. Aldosterone appears to regulate ENaC activity rather than abundance (Kemendy et al., 1992; Kleyman et al., 1992; Eaton et al., 1995; Ling et al., 1997) and acts to increase sodium, and thus water, reabsorption within the CCD and OMCD.

In addition to the quantification of transporters and channels expressed in transplanted metanephroi, the number of tubules present was also determined. Non-biased stereological counting methods (Nyengaard and

Bendtsen, 1992; Nyengaard et al., 1994) were employed to determine glomerular, and hence nephron, number in the metanephroi. In order to support the glomerular counts, and provide further insight into the stage of nephrogenesis of transplanted metanephroi, angiotensin II type 2 receptor (AT_2R) expression was investigated. Despite its function being largely unknown, AT_2R is thought to play a role in the apoptotic process during nephrogenesis (Wolf, 2002; Wolf, 2005). AT_2R is highly expressed in the developing kidney, dropping to barely detectable levels in adulthood, appearing to mirror the nephrogenic state of the kidney (Kakuchi et al., 1995; Tufro-McReddie et al., 1995). For this reason, AT_2R was chosen as a marker of renal development, since a lack of AT_2R expression would give a further indication that nephrogenesis had ceased within the transplants.

3.1.2 Aims of this study

The first aim of this part of the study was to estimate glomerular, and thus nephron, number within transplanted metanephroi using non-biased stereology. Due to their comparable sizes, embryonic day 21 (E21) and post-natal day 1 (PND1) Lewis rat kidneys were used as controls for the estimation of the number of glomeruli in transplanted metanephroi. By comparing nephron number in transplanted metanephroi with E21 and PND1 kidneys it should be possible to determine whether the limited growth of the transplants was accompanied by a reduction in nephron number.

The second aim of the study was to assess the maturity of the tubules. Tubular maturity, particularly in terms of the urine concentrating process, was evaluated by analysing the expression of a range of transporters and channels (listed above) central to the urine concentrating mechanism by immunohistochemistry, reverse transcriptase PCR (RT-PCR) and quantitative real time-PCR (qPCR). Comparing mRNA and protein expression within the transplanted metanephroi with E21 and PND1 rats, together with adult rats, allowed the assessment of tubular maturity of the transplants in terms of their potential ability to concentrate urine. These data, together with the results of the glomerular counts, and the *in vivo* data, also helped to determine an 'effective age' of the transplants up to four months post-transplantation.

3.2 Materials and Methods

All experiments described herein were performed under the authority of a Home Office Project Licence (PPL numbers 40/2717 and 40/2455).

3.2.1 Animals

The animals employed in this study were Lewis rats (Charles River, UK) which were housed in the Biological Services Unit (BSU) at the University of Manchester. Animals were maintained in a 12-hour light/dark cycle with free access to water and food (Bantin and Kingman rat and mouse expanded diet, Hull, UK). All E21 and neonatal rats were obtained from time-mated female Lewis rats (Charles River, UK).

3.2.2 Explantation of renal tissue and perfusion fixation

Adult female rats were anaesthetised with the sodium barbiturate, Inactin (100 mg/kg) and a midline incision was performed. The abdominal aorta was identified, a small incision made and polyethylene tubing (0.63mm internal diameter, 1.40mm outside diameter) (Smiths Medical, Kent) was advanced towards the kidneys and tied into place with cotton thread. The aorta was tied off, with cotton thread, above the level of the kidneys and 0.01M phosphate-buffered saline (PBS) (Sigma-Aldrich, UK) was perfused at a rate of 15mL/min for 2 minutes to rinse away any blood from the

kidneys. Both renal veins were severed to allow the PBS to drain away. Following this infusion, the kidneys were perfusion-fixed with 4% Paraformaldehyde (PFA, Sigma-Aldrich, UK) at a rate of 15 mL/min for 2 minutes. Transplanted metanephroi, to be processed for immunohistochemistry, were perfusion-fixed in an identical manner, but following clearance study with the animal already anaesthetised. Transplants to be embedded for glomerular counting were immersion-fixed in PFA overnight at 4 °C following explantation at either 3 weeks post-transplantation or at the end of clearance study, following euthanisation of the host adult rat by Schedule 1 procedures (Home Office UK).

Embryonic day 21 rats (E21) were obtained following Isoflurane (Abbott Laboratories Ltd, Kent) anaesthesia (flow rate 1 L/min O_2, 2.5% Isoflurane) of the mother. A midline laparotomy was performed and each embryo was dissected free from the uterine horn. A midline laparotomy was performed on each embryo and the E21 kidneys were identified and dissected free from surrounding tissue. E21 kidneys were immersion-fixed in 4% PFA overnight at 4 °C. Postnatal day 1 (PND1) pups were anaesthetised with Isoflurane and all renal mass was explanted in a similar manner to that described for E21 kidneys. PND1 kidneys were immersion-fixed in 4% PFA overnight at 4 °C in readiness for wax embedding. All animals were euthanased by Schedule 1 methods (Home Office, UK).

3.2.3 Paraffin wax embedding

All renal tissue was embedded in wax using a Shandon Citadel 2000 automatic processing machine (Thermo Electron Corporation, USA). Tissue was removed from the machine and placed onto heated trays in an LTE Qualivac oven (LTE Scientific Ltd, UK). Air was evacuated from the oven until a pressure of 25 mm Hg was reached. Tissue was left for 30 minutes to allow the wax to fully penetrate the tissue. This process was repeated with fresh wax and finally the tissue was embedded into moulds. All tissue was stored at room temperature.

3.2.4 Glomerular counts

3.2.4.1 Tissue sectioning for glomerular counts

Transplanted metanephroi and kidneys from E21 and PND1 were explanted, fixed and embedded as described previously. Prior to sectioning, embedded tissue was placed at –20 °C for around 10-15 mins. All kidneys were sectioned on an Anglia Scientific microtome (Cambridge, England). A random starting position was chosen (e.g. the 4^{th} section cut into the kidney) and then this section was taken together with the next parallel (look up) section. This was termed a section pair. Every 'n th' section and parallel section, was then taken throughout the block until the kidney was completely sectioned. For example if the 'n th' section was 20, every 20^{th} section pair would be taken. Sections were cut at 10 µm

thickness. Sections were floated on a JB2 water bath (Grant instruments, Cambridge) at 37 °C and mounted onto twin-frosted slides (Scientific Laboratory Supplies, Manchester). Slides were left to dry on a hot plate (Laboratory Thermal Equipment, Greenfield) for 10 minutes before being left to cool overnight. All slides were stored at room temperature.

3.2.4.2 Haematoxylin and Eosin staining for glomerular counts

Slides were de-waxed with xylene for 15 mins before being immersed in a graded series of industrial methylated spirit (IMS, Genta Medical, York) washes. Slides were washed under running tap water before being stained with haematoxylin (VWR International, Poole) for 2 mins followed by eosin (VWR International, Poole) for around 15 secs. Slides were washed with 95% ethanol (VWR International Ltd, Poole) and following further graded IMS washes, sections were immersed in xylene for 12 mins. All sections were mounted using Eukitt mounting solution (Sigma-Aldrich, UK).

3.2.4.3 Estimation of glomerular number

Glomeruiar numbers in every nth 10 µm kidney section pair were estimated using a modified version of the physical fractionator technique (Sterio, 1984; Nyengaard and Bendtsen, 1992; Armitage et al., 2005) for transplanted metanephroi, E21 and PND1 kidneys. Briefly, mature vascularised glomeruli were counted using a Medilux-12 microscope (Kyowa, Japan) within a frame on one kidney section and then compared

to a second 'look-up' section on the corresponding part of the parallel kidney section (see figure 3.2).

Glomeruli that were present on both the initial section and parallel 'look-up' section were discounted. The counting frame was then moved and the process repeated until the whole kidney section was counted. 8-10 section pairs were counted for each sample.

The final number of glomeruli was estimated using the following formula:

$$\text{Total number of glomeruli} = (\Sigma Q \times 1/n) \times 2$$

where Q = number of glomeruli counted, n = fraction of section pairs counted, x 2 = estimate is doubled since glomeruli are counted 2 ways, initial section versus 'look-up section and vice-versa.

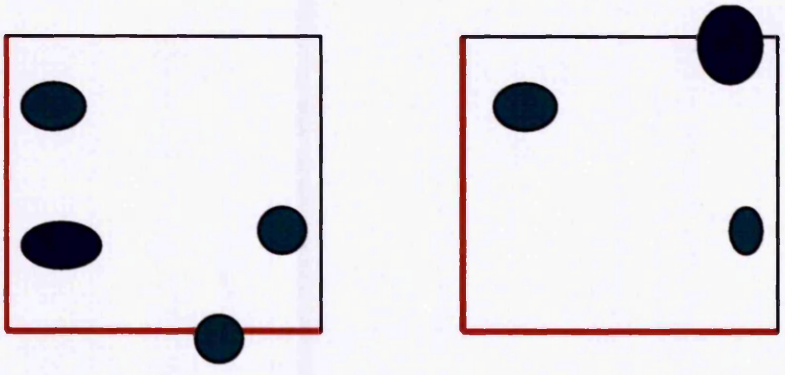

Figure 3.2. Schematic showing how glomeruli were counted. Schematic shows an initial section (left-hand box) and a parallel 'look-up' section (right-hand box). Circles denote glomeruli. A glomerulus was discounted if it touched the forbidden line (red line). Glomeruli that appeared in both initial and look-up section were also discounted. Only glomeruli that appeared in the initial and not the look-up section were counted and vice-versa. The glomeruli that would be counted in this illustration appear as dark blue above.

3.2.5 Immunohistochemical staining

3.2.5.1 Tissue sectioning for Immunohistochemistry

Prior to sectioning, embedded tissue was placed into a -20 °C freezer for 10-15 mins. 5 μm sections of E21, PND 1,7,14 and adult kidneys were cut using an Anglia Scientific microtome (Cambridge, England). Sections were floated on a JB2 water bath (Grant instruments, Cambridge) at 37 °C and mounted onto superfrost plus slides (VWR International, Poole). Slides were left to dry on a hot plate (Laboratory Thermal Equipment, Greenfield) for 10 minutes before being left to cool overnight. All slides were stored at room temperature.

3.2.5.2 Immunohistochemistry

All slides were placed in xylene (Genta Medical, York) overnight to de-wax at room temperature. Sections were rehydrated through immersion in a graded series of ethanol washes (99%, 96%, 70%) (VWR International, Poole). Any endogenous peroxidase was blocked by 30% hydrogen peroxide (Sigma-Aldrich, UK) in 100% methanol (VWR International, Poole). Antigen retrieval was performed by immersing slides in 0.05M TEG buffer at pH 9 (Sigma-Aldrich, UK) and microwaving on full power until the boiling point was reached. TEG buffer was kept softly boiling for 7 minutes. Following immersion in 50mM NH_4Cl (Sigma-Aldrich, UK) in 0.01M PBS for 30 minutes, slides were washed in solution A (1% BSA,

0.05% saponin and 0.2% gelatine) three times. All primary antibodies (table 3.1) were raised in rabbit and diluted in 0.1% BSA with 0.3% Triton X-100 (Sigma-Aldrich, UK) at the dilutions listed in table 3.1. 100 µl of diluted primary antibody was applied to each slide. Slides were placed in a humidity chamber and left overnight at 4 °C. Following incubation, slides were washed three times in solution B (0.1% BSA, 0.05% saponin and 0.2% gelatin). Slides were incubated with swine anti-rabbit Immunoglobulin (Dako Ltd, Cambridge), labelled with horseradish peroxidase, at a dilution of 1:100 in 0.1% BSA with 0.3% Triton X-100. Incubation was for 1h at RT. As negative controls, some slides were incubated with 0.1% BSA with 0.3% Triton X-100 only rather than primary or secondary antibody. Following three more washes with solution B, sections were incubated in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, UK) and observed under a Medilux-12 microscope (Kyowa, Tokyo, Japan) until staining appeared. At this point, DAB was removed via a series of 0.01M PBS washes. Sections were counterstained with Mayers haematoxylin (Sigma-Aldrich, UK) for 15 s. Sections were dehydrated with a graded series of ethanol washes and immersed in xylene. All sections were mounted using Eukitt mounting solution (Sigma-Aldrich, UK) and examined and photographed using a Leica microscope (Leica Microsystems, UK) and digital camera.

Target protein	Dilution used	Supplier
AQP1	1:200	Dr David Marples*
AQP2	1:500	Dr David Marples*
AT_2R	1:50	Abcam
ENaC α-subunit	1:200	Sigma-Aldrich
NKCC2	1:200	New England Biolabs
UT-A1/2	1:100	Dr Craig Smith*
UT-A1/3	1:100	Dr Craig Smith*

Table 3.1. Antibodies employed in immunohistochemical studies. All primary antibodies were raised in rabbit. * antibodies were non-commercially produced and were a kind gift from either Dr David Marples (Leeds) or Dr Craig Smith (Manchester).

The specificity of all the above antibodies have been shown in previous studies, as follows; AQP1 and 2 (Li et al., 2001; Shaw and Marples, 2002), AT_2R (Ruiz-Ortega et al., 2003), ENac α subunit (Zhou et al., 2006), NKCC2 (Schmitt et al., 2003) and UT-A1/2 and UT-A 1/3 (Fenton et al., 2002; Fenton et al., 2006).

3.2.6 mRNA expression of target genes

3.2.6.1 Obtaining tissue for RNA extraction

Kidneys and/or transplanted metanephroi were removed from female adult Lewis rats, E21 and PND1 Lewis rats following euthanisation of the animal

by Schedule 1 methods, as described previously. All tissue was immediately snap frozen in liquid nitrogen (BOC group, UK) following removal and was stored at -80 °C until required for use.

3.2.6.2 RNA extraction

Up to 100 mg of tissue from E21, PND1, transplanted metanephroi and adult kidneys was homogenised in 1 mL of trizol (Invitrogen, UK) and incubated for 5 mins at RT. 200 µl of phenol chloroform (VWR International, Poole) was added and each tube was shaken vigorously before being left to stand for 2-3 mins at RT. Each sample was centrifuged at 4 °C for 15 mins at 12,000x g using a Jouan 2022 centrifuge (Thermo electron corporation, UK) and the upper aqueous phase was decanted into a separate tube. 500 µl of isopropanol (Sigma-Aldrich, UK) was added before a further centrifugation at 4 °C for 10 mins at 12,000x g. All samples were washed with 1 mL 75 % ethanol (VWR International, Poole) before a final centrifugation at 4 °C for 5 mins at 7,500x g. Each RNA pellet was air dried for 10 mins before being redissolved in a known quantity of RNAse free water (Invitrogen, UK). 2µl of this RNA, diluted 1:100 in RNAse free water was then analysed on a Beckman DU-530 UV/VIS spectrophotometer (Beckman Instruments Inc, Fullerton, CA,

USA) by plotting a curve of the absorption at 260 and 280nm. The value at 260nm was then placed into the following formula:

Total RNA in sample (μg) = A_{260} x dilution x volume (μl) x 40 (ribosomal RNA)

This enabled RNA to be diluted appropriately so that each sample contained 1 μg RNA per 1 μl of sample. Quality of RNA was confirmed by means of an RNA denaturing gel. Briefly, 5 μl of sample was mixed with 1 μl ethidium bromide (Sigma-Aldrich, UK) and 15.5 μl denaturing solution consisting of formamide, formaldehyde, 20x MOPS and DEPC H_2O (all Sigma-Aldrich, UK). Each sample was loaded onto a 2% agarose gel containing DEPC H_2O, 20x MOPS and formaldehyde and ran at 72V for 6 hours. An example of this RNA denaturing gel can be seen in Figure 3.3.

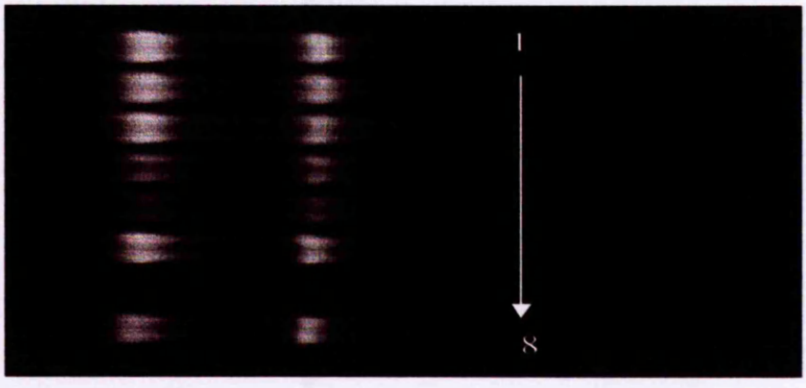

Figure 3.3. Example of an RNA denaturing gel. Gel was run at 72V for 6 hours to assess the quality of total RNA. Lane 7 has a lack of expression indicating a lack of total RNA, lane 5 has a reduced expression of RNA which may be indicative of the poor quality of that particular sample. As such both samples would be discounted. All other lanes show strong expression of denatured RNA indicating that the total RNA is of sufficient quality.

3.2.6.3 Conversion of total RNA to cDNA

1 µg of RNA per sample was treated with 1 µl 10x DNase buffer, 1U DNAse (both Eurogentec, UK) and made up to 10 µl with RNAse free water (Invitrogen, UK). Samples were incubated at RT for 15 mins before 1 µl 25mM EDTA (Eurogentec, UK) was added. First strand cDNA synthesis was performed by the addition of 2 µl of 200 ng/mL random hexamer primer (Roche pharmaceuticals, UK) and 2 µl of a 10mM deoxynucleoside triphosphate (dNTP) mix (Bioline, UK). Following incubation at 65 °C for 5 mins, samples were chilled on ice for 2-3 mins. 4 µl of 5x First strand buffer and 2 µl 0.1 M DTT (both Eurogentec, UK) were added and following incubation at RT for 10 mins, 1 µl SuperScript II RT (200 U, Invitrogen, UK) was added to each tube. Samples were incubated for 50 mins at 42 °C in a Techne hybridiser HB-1D (Barloworld Scientific Limited, UK) followed by a 15 min denaturation at 70 °C. The resultant cDNA templates were stored at -20 °C. cDNA was tested for quality by running a 2% agarose gel at 120V for around 45 mins, an example of which can be seen in Figure 3.4.

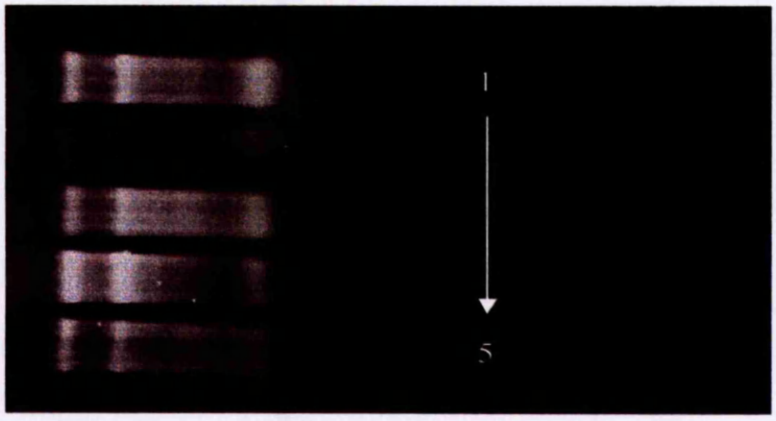

Figure 3.4. Example of a gel to assess the quality of cDNA. A 2% agarose gel, ran at 120V for 45 mins. Lanes 1, 3, 4 and 5 indicate that cDNA is good quality with one distinct smear. Lane 2 shows less expression which may be indicative of poor quality cDNA. As such this sample would be discounted.

3.2.6.4 Taqman qPCR

Taqman quantitative RT-PCR (qPCR) is based on the principle that a probe specific to a certain gene sequence is labelled with a fluorescent tag. Thus, once the probe binds to its target sequence, following the binding of specific primers, fluorescence is emitted as the gene product is transcribed. Thus, more gene product is proportionate to more fluorescence emitted. This fluorescence is read by the ABI prism 7000 software system (ABI instruments, California, USA). This software calculates the number of PCR cycles required to reach the maximum rate of transcription of gene product, known as cycle time (Ct). A typical graph for this can be seen in figure 3.5.

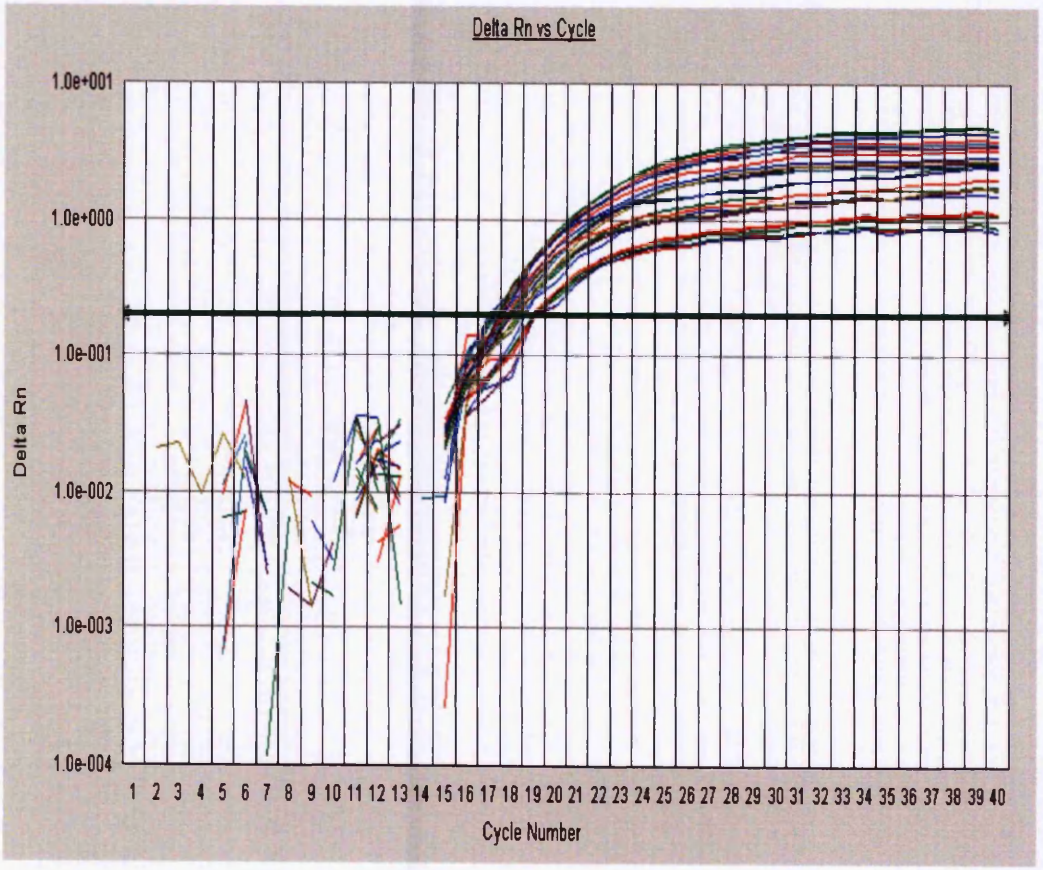

Figure 3.5. Example of a fluorescence curve from a qPCR run. The graph shows fluorescence emitted by the Taqman probe upon binding and transcription of a specific gene product. This fluorescence is measured by the ABI Prism 7000 software system. Shown on the graph is number of PCR cycles (x axis) and fluorescence (y axis). In the example above, it has taken around 14-15 PCR cycles for fluorescence to reach measurable levels, and 19-20 PCR cycles to reach maximum fluorescence-emission rates (shown by the exponential part of the curves). Thus cycle time (Ct) would be in the region of 19-20 for this particular gene.

All primers and Taqman probes were designed using the primer express software v2.0 (ABI Instruments, California) and were ordered from Eurogentec (Southampton, UK). These are listed in table 3.2.

ß-actin	Forward primer 5' GACAGGATGCAGAAGGAGATTACTG 3' Reverse primer 5' GAGCCACCAATCCACACAGA 3' Taqman probe 5' CACCATGAAGATCAAGATCATTGCTCCTCCT 3'
AQP1	Forward primer 5' AGTGTGTGGGAGCCATCGTT 3' Reverse primer 5' GGTCATTTCGGCCAAGTGAGT 3' Taqman probe 5' CGGCATCACCTCCTCCCTGCTC 3'
AQP2	Forward primer 5' GCCCTCTCCATTGGTTTCTCT 3' Reverse primer 5' GGCTGGATTCATGGAGCAA 3' Taqman probe 5' CCTGGGCCACCTCCTTGGGATCT 3'
AT_2R	Forward primer 5' AATTACCCGTGACCAAGTCTTGA 3' Reverse primer 5' AGAACATGGAAGGGAAGCCA 3' Taqman probe 5' AGCTGCTGTTGTGTTGGCATTCATCATT 3'
ENaC*	Forward primer 5' GCCTTGTAGTGTGATCAACTACAAACT 3' Reverse primer 5' CCAATCCTGGGACTTCACAGAT 3' Taqman probe 5' TCTGCCGGCTACTCACGGTGGC 3'
NKCC2	Forward primer 5' ACAGGAGGACCCATGACAAGA 3' Reverse primer 5' GCAGCAGATACAGAGGCCACTA 3' Taqman probe 5' TCCTGGACATAACCCATGCCTTTACGAAGA 3'
UT-A1/2	Forward primer 5' TGGAGCCCTCAATGAGATGTG 3' Reverse primer 5' CAAAGAGCCATCGGTGGGTAT 3' Taqman probe 5' AGCGCTCATCAAAGCCACGCTAGTTTAAA 3'
UT-A1/3	Forward primer 5' CCCTACCTGGCCTTCAAGCT 3' Reverse primer 5' TCCTTTTCTTCAGGCATTTCCA 3' Taqman probe. 5' CCAGGATACACACCCAGCCCTTCCC 3'

Table 3.2. Primer and Taqman probe sequences used for qPCR. * denotes primers and probes were targeted to the α-subunit of ENaC.

Prior to analysis of samples, all primers and probes were optimised as follows:

For primer optimisation, the following combinations of primer concentrations were used (forward/reverse primers): 50/50 nM, 50/300 nM, 50/900 nM, 300/50 nM, 300/300 nM, 300/900, 900/50 nM, 900/300 and 900/900 nM. For probe optimisation, concentrations of 50,100,150 and 200 nM were used.

To each well of a 96-well plate (ABI Prism, California, USA), 2 µl of cDNA template was added together with 2 µl of one of the primer concentration

combinations. 1 µl of Taqman probe (150 nM) was added, followed by 12.5 µl of master mix solution, which consists of 10x reaction buffer, 50mM $MgCl_2$, 5mM dNTP solution and 0.025 U/µl Hot Goldstar Taq (Eurogentec, Southampton, UK) and 7.5 µl RNAse free dH_2O (Invitrogen, UK). Each primer pair combination was run in triplicate. An optical cover (ABI Prism, California, USA) was applied and the plate was spun at 2000x g for 2 mins using an Eppendorf centrifuge 5810 (Eppendorf, Cambridge, UK). Each qPCR plate was run on the ABI Prism 7000 software system (ABI Prism, California, USA) for 40 cycles. Primer pairs giving the lowest but stable cycle time (Ct) values were deemed the optimum primer concentrations and were employed in future experiments. For Taqman probe optimisation, optimum concentrations of primers were used and probe concentrations of 50, 100, 150 and 200 nM were employed. All Taqman probe concentrations were run in triplicate. The probe concentration that gave the lowest but stable Ct values was chosen for use in later experiments. Optimum primer and probe concentrations for each target gene are shown in table 3.3.

Target gene	Forward primer (nM)	Reverse primer (nM)	Probe (nM)
ß-actin	300	900	150
AQP1	300	900	150
AQP2	900	900	150
AT_2R	300	900	150
ENaC	900	300	150
NKCC2	300	900	100
UT-A1/2	300	900	150
UT-A1/3	300	300	100

Table 3.3. Optimum concentrations of primers and probes for qPCR.

cDNA templates from adult Lewis rat kidneys (control), E21 and PND1 Lewis rat kidneys and transplanted metanephroi were analysed for each target gene and reference gene (β-actin). Each sample was run in triplicate on 96 well plates as described above. Optimum primer and probe concentrations were used and plates were analysed on the ABI prism 7000 software. Wells without either cDNA template, primer sets or Taqman probe were run as negative controls. Gene expression was calculated as described below.

3.2.6.5 Calculation of gene efficiencies and relative gene expression

In order to calculate relative gene expression, the ΔΔCt method was used (Livak and Schmittgen, 2001), as shown below:

$2^{-\Delta\Delta Ct}$ where ΔΔCt is described as:

$$\Delta \text{ Ct } (Ct_{\text{target gene}} - Ct_{\text{reference gene}}) - Ct_{\text{target gene of control (adult rat cDNA)}}$$

In order to test the gene efficiencies of both target gene and reference gene (β-actin), the following volumes of adult rat cDNA were loaded in triplicate; 0.25, 0.5, 1, 2, 4 and 8 µl. By plotting cDNA volume loaded against Δ Ct ($Ct_{\text{target gene}} - Ct_{\text{reference gene}}$), it was possible to calculate whether the gene efficiencies of target and reference genes were approximately equal. A gradient of less than 0.1 meant the ΔΔCt method for calculating relative gene expression could be employed. An example of a graph of this gene efficiency check can be seen in figure 3.6.

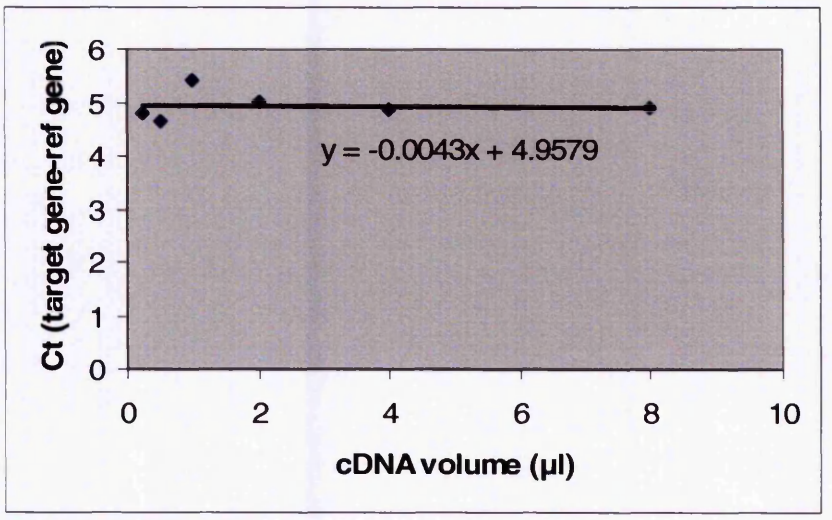

Figure 3.6. Example of a graph showing gene efficiency check. Values are calculated by subtracting the Ct value of the reference gene (ß-actin) from the Ct value of the target gene (in this case UT-A1,2) at cDNA volumes of 0.25, 0.5, 1, 2, 4 and 8 µl. A linear trendline is then added, and a gradient less than 0.1 (in this case 0.004) means that the $2^{-\Delta\Delta Ct}$ method of calculating gene expression can be employed, since the gene efficiencies of target and reference gene are very similar.

3.2.6.6 Reverse transcriptase PCR

In addition to qPCR, reverse-transcriptase PCR (RT-PCR) of samples was also run. Primers for AQP 1 and 2, AT_2R, ENaC, NKCC2, UT-A1/2, UT-A1/3 and β-actin were the same as those listed in table 3.2. 1 µl of both forward and reverse primers (both at 20 µM) were added to 2 µl cDNA from either adult, E21 or PND1 Lewis rat kidneys or transplanted metanephroi. 41.5 µl of PCR master mix solution (Eurogentec, Southampton, UK), composed of 5 µl 10x reaction buffer, 4 µl 25mM $MgCl_2$, 1.75 µl 2.5mM dNTP solution and 31.5 µl RNAse free H_2O (Invitrogen, UK), was added together with 2.5 µl of 0.025 U/µl Taq

polymerase (Bioline, UK). Wells with either no cDNA or primers were run as negative controls (not shown). The PCR reaction was run for 30 cycles on a PTC-220 thermal cycler (MJ Research Inc, California, USA). Following the PCR reaction, samples were loaded onto a 0.01M Tris-borate buffer (TBE) 2% agarose gel and run at 120V for approximately 45 mins. All samples were visualised and photographed under UV light using a Syngene gene genius 3088S bio imaging system (Syngene, Cambridge, UK).

Following the RT-PCR reaction, the PCR product was purified using a Qiagen PCR purification kit (Qiagen Ltd, Crawley, UK). 4 µl of this product was added to 2 µl of 3.2 µM forward primer (Eurogentec, UK) specific to the target gene, 2 µl 5x sequencing buffer (Qiagen, UK), 1 µl Big dye reaction buffer (Qiagen, UK) and 1 µl RNAse free H_2O (Invitrogen, UK). A PCR reaction was run for 30 cycles on a PTC-220 thermal cycler (MJ Research Inc, California, USA). To remove unincorporated nucleotides from the reaction 1.5µl of 3 M sodium acetate pH 5, 1 µl of glycol blue and 30 µl of 100% ethanol was then added to precipitate the reaction and incubated overnight at -20°C. Following this, the sample was spun at 16000 x *g* for 20 minutes and the supernatant decanted. The pellet was washed with 500 µl of 75 % ethanol and spun for a further 10 minutes and dried for 10 minutes at room temperature. The pellet sample was then run on an ABI sequencing gel in the University of Manchester sequencing unit to check that the PCR product was the correct target sequence.

3.2.7 Statistical analysis

Data are presented as the mean ± SEM. All statistical analyses were carried out using the Statistical Package for Social Sciences version 13.0 (SPSS 13.0 for Windows). Statistical analysis was by one-way ANOVA for glomerular counts and qPCR data. Statistical significance was set at the 5 % level.

3.3 Results

3.3.1 Glomerular counts

The estimate of glomerular number (Figure 3.7) showed that there were, on average, around 4200 glomeruli per transplanted metanephros. This value was significantly higher than the estimate for E21 Lewis rat kidneys ($P = 0.006$) but significantly lower than that for PND1 animals ($P = 0.001$). Estimates of glomeruli in PND1 kidneys were also significantly higher than in E21 kidneys ($P = 0.001$).

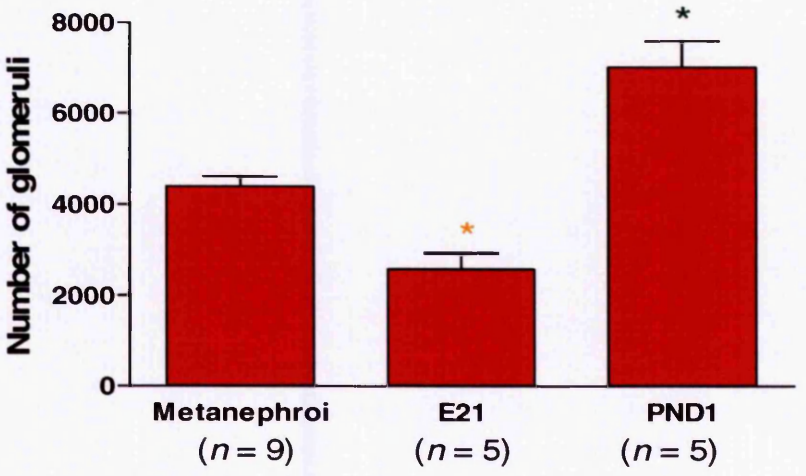

Figure 3.7. Estimation of glomerular number. Glomeruli were counted using a non-biased stereological counting technique within transplanted metanephroi (3 weeks to 4 months post-transplant), embryonic day 21 (E21) and post-natal day 1 (PND1) Lewis rat kidneys. Statistical analysis was by one-way ANOVA. $P < 0.05$ was deemed significant. * $P = 0.006$ compared to metanephroi, * $P = 0.001$ compared to both metanephroi and E21 Lewis rat kidneys.

3.3.2 Immunohistochemical localisation of target proteins

For all of the following target proteins, 2 negative control samples were used, one slide with primary antibody only and one with secondary antibody only. In each case, for all target proteins examined, no staining was observed in either of the negative control slides. However, only one of these controls is shown for each target protein in the figures that follow.

3.3.2.1 Urea transporters 1 and 2 (UT-A1 and 2)

UT-A1 was localised, by immunohistochemistry, within the inner medullary collecting ducts (IMCD) of adult rat kidneys whilst UT-A2 was localised within the descending thin limb of the loop of Henle (Fig 3.8A-B). Staining of UT-A1 was no longer apparent within the IMCD when slides were incubated in primary antibody only (Fig 3.8C). Staining for UT-A1 and 2 was not visible in transplanted metanephroi (Fig 3.8D), E21 (fig 3.8E) or PND1 kidneys (Fig 3.8F).

3.3.2.2 Urea transporters 1 and 3 (UT-A1 and 3)

UT-A1 and 3 were localised within the inner medullary collecting ducts (IMCD) of adult rat kidneys by immunohistochemistry (Fig 3.9A). Sections that were incubated in the absence of secondary antibody showed a lack of staining (Fig 3.9B). Immunohistochemistry showed UT-A1/3 was not localised within transplanted metanephroi (Fig 3.9C-D), E21 (Fig 3.9E) and PND1 kidneys (Fig 3.9F).

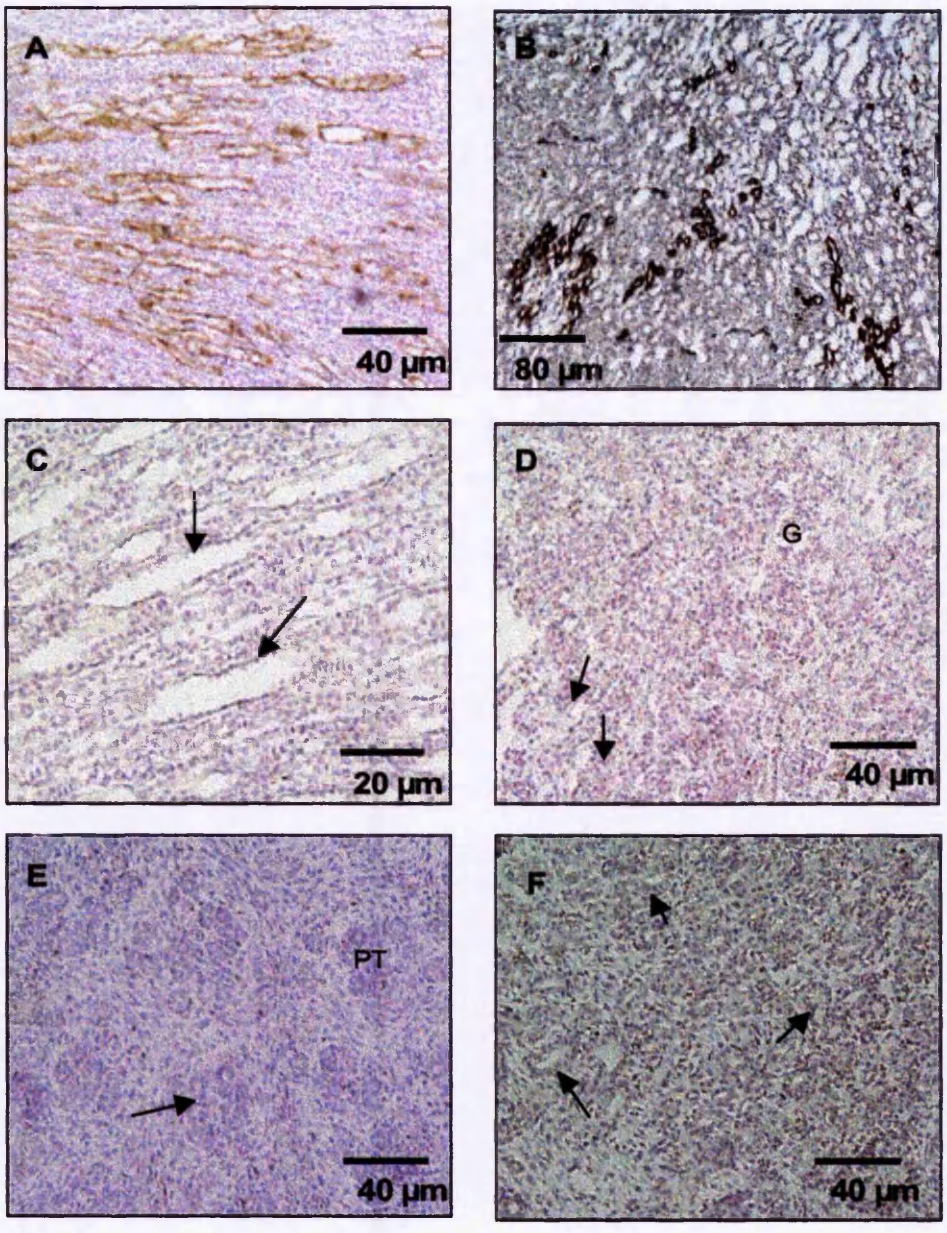

Figure 3.8. Immunohistochemical localisation of UT-A1 and 2. Intense staining is shown in the collecting ducts of the inner medulla (A) and in the descending thin limbs of the loop of Henle (B) of adult control sections. A section that had no secondary antibody applied is shown within the collecting duct region (arrowed) (C). Immunohistochemistry failed to show any staining in the collecting ducts (arrowed) of transplanted metanephroi (D), embryonic day 21 (E21) kidneys (E) or post-natal day 1 (PND1) animals (F). G denotes glomerulus; PT, proximal tubules. Original magnifications for A, D, E and F are x100, x50 for B and x200 for C.

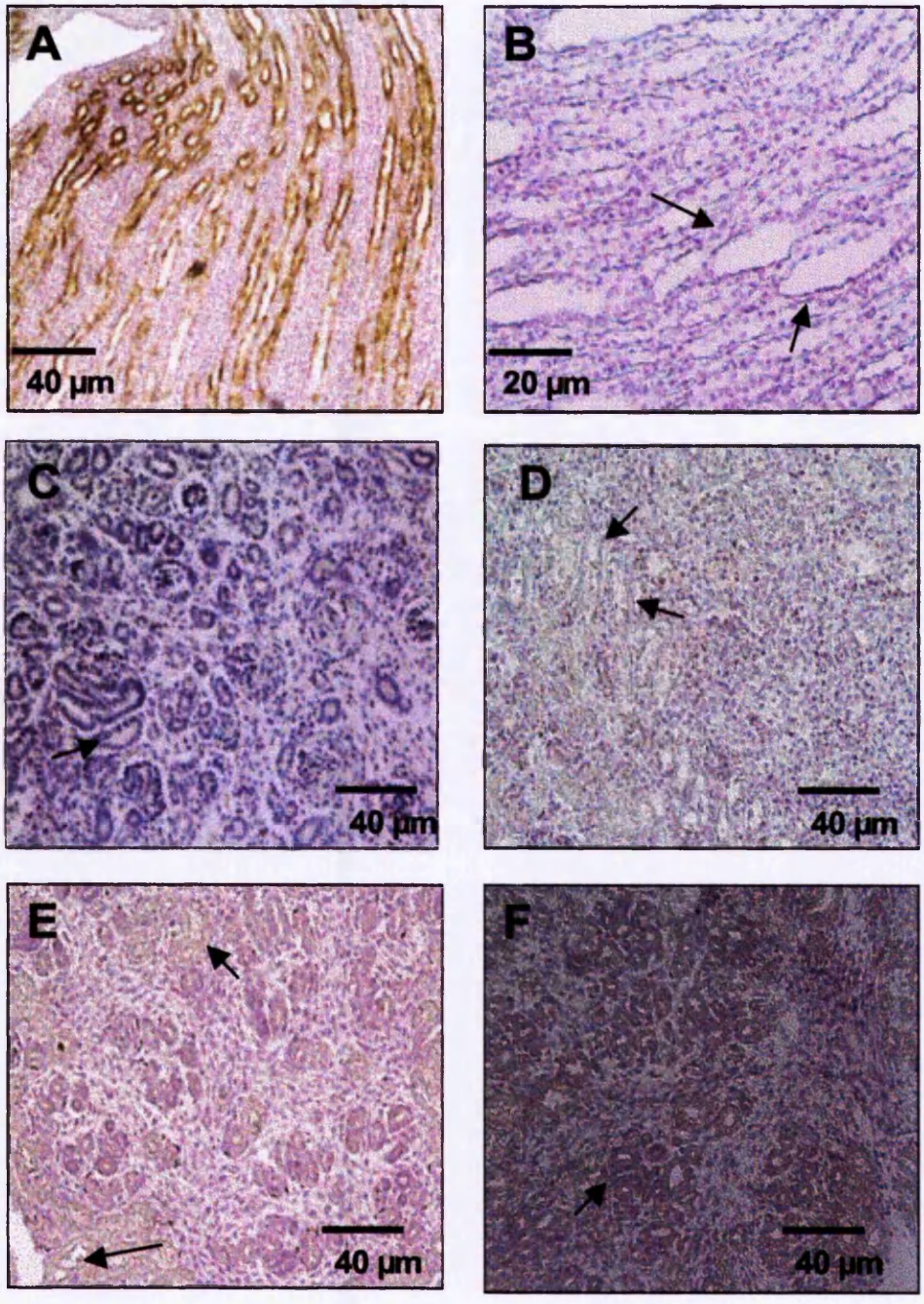

Figure 3.9. Immunohistochemical localisation of UT-A1 and 3. Intense staining is shown in the collecting ducts of the inner medulla of adult rat controls (A) but not in sections (B) that had no secondary antibody applied (arrowed). Immunohistochemistry failed to show any staining within collecting ducts (arrowed) of transplanted metanephroi (C-D), embryonic day 21 (E21) kidneys (E) or post-natal day 1 (PND1) animals (F). Original magnifications for A, C, D, E and F are x100 and x200 for B.

3.3.2.3 *Aquaporin 1 (AQP1)*

Intense staining was apparent within proximal tubules, thin descending limbs (tDL) and descending vasa recta (DVR) of control adult rats (Fig 3.10A) but was not present in negative controls (Fig 3.10B). Staining for AQP1 was far less intense within transplanted metanephroi (Fig 3.10C-D) but there was localised staining visible in proximal tubules, tDLs and DVR. Staining appeared more intense within both E21 and PND1 animals (Fig 3.10E-F) compared to the transplants with localised staining apparent within proximal tubules, tDLs and DVR.

3.3.2.4 *Aquaporin 2 (AQP2)*

Aquaporin 2 was also expressed in all renal tissue tested but within the collecting ducts only. Staining appeared most intense in adult positive controls (Fig 3.11A), with negative controls again failing to show any staining (Fig 3.11B). In contrast, staining in transplanted metanephroi, though localised to the collecting ducts, appeared to be more disparate compared to the other groups (Fig 3.11C-D). E21 and PND1 Lewis rats also showed intense staining for AQP2 with strong localised staining visible on the luminal side of the collecting ducts (Fig 3.11E-F).

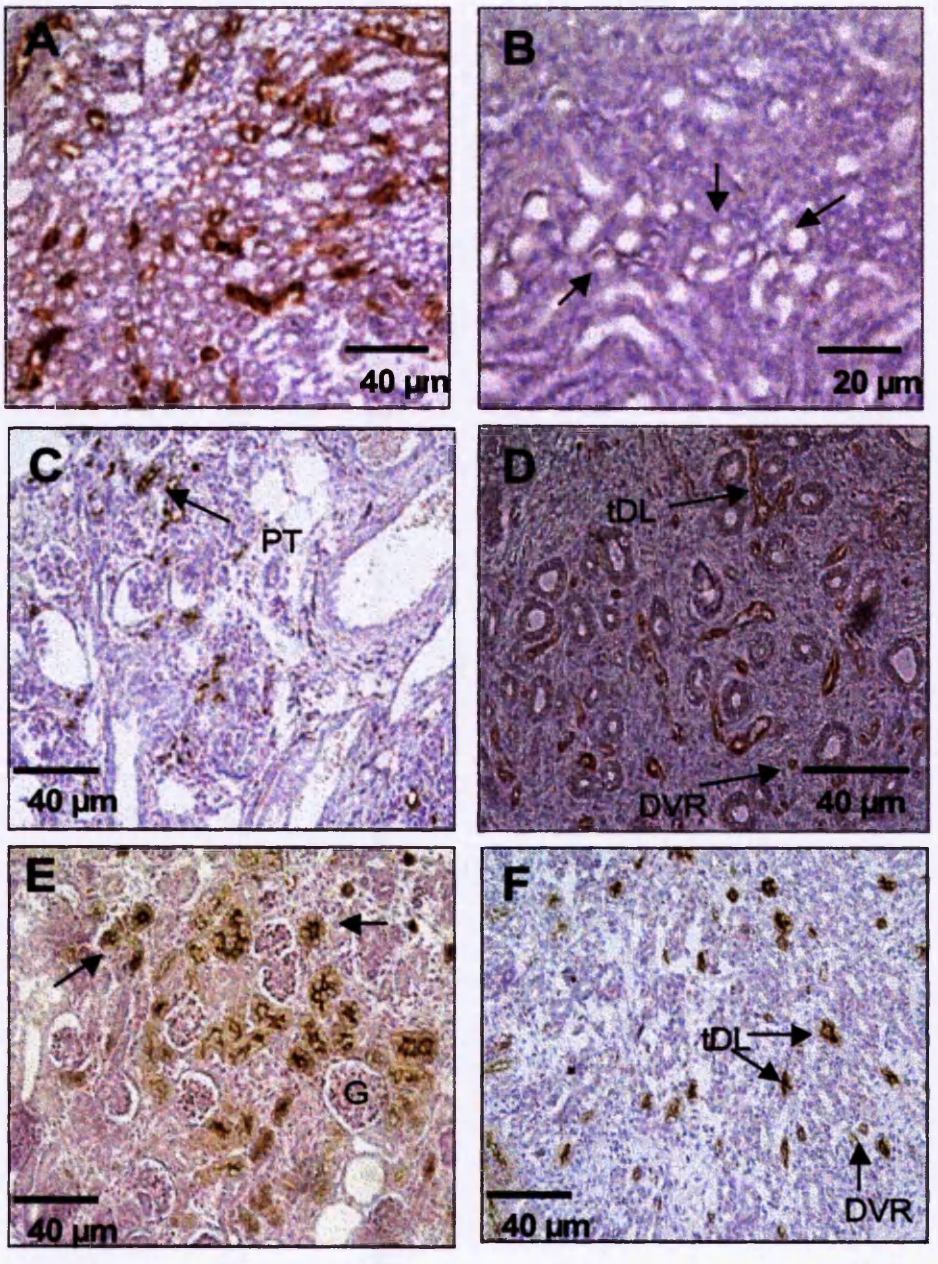

Figure 3.10. Immunohistochemical localisation of AQP1. Intense staining is shown in the tubules of adult rat controls (A) but not in sections (arrowed, B) that had no secondary antibody applied. Staining is apparent in transplanted metanephroi in proximal tubules (PT) in (C) and thin descending limbs of the loops of Henle (tDL) and descending vasa recta (DVR) in (D). Intense staining is visible in embryonic day 21 rats in proximal tubules (arrowed) close to the glomeruli (G) in (E) and in thin descending limbs and DVR of post-natal day 1 animals (F). Original magnifications for A,C,D,E and F are x100 and x200 for B.

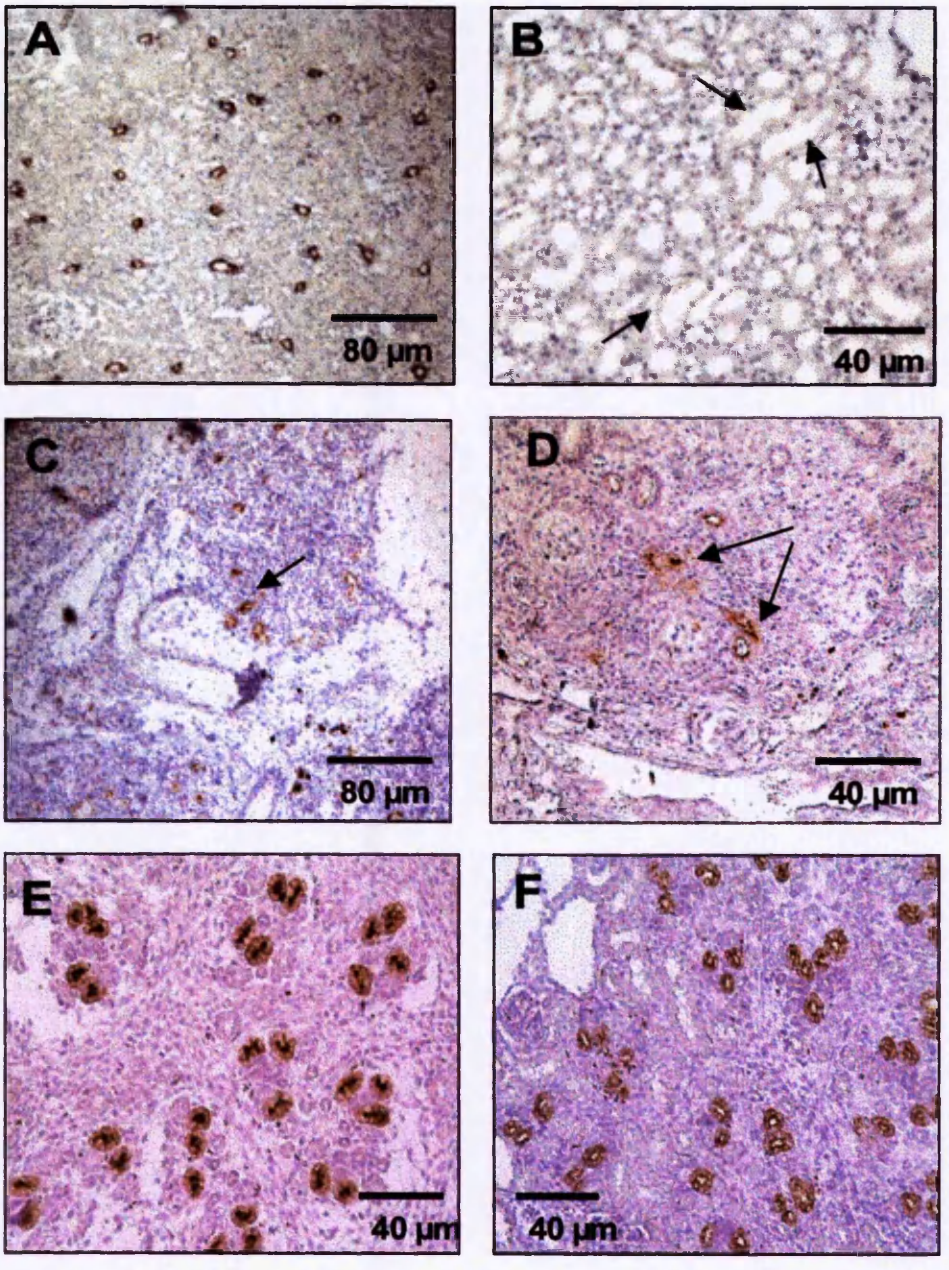

Figure 3.11. Immunohistochemical localisation of AQP2. Intense staining is shown in the collecting ducts of adult rat controls (A) but not in collecting ducts of sections (arrowed, B) that had no primary antibody applied. Staining is apparent in transplanted metanephroi in collecting ducts (arrowed) in (C) and (D). Intense staining is also visible in collecting ducts of embryonic day 21 rats (E) and post-natal day 1 animals (F). Original magnifications for A and C are x50. B,D,E and F are x100.

3.3.2.5 Epithelial Na channel (ENaC)

ENaC was intensely stained and localised to the collecting ducts of the cortex and outer medulla in adult positive controls (Fig 3.12A). This staining disappeared when primary antibody was not applied (Fig 3.12B). Staining within transplanted metanephroi was far less widespread than within positive controls but there was staining visible within the collecting ducts of the medulla (Figs 3.12C-D). E21 animals appeared to show a similar amount of staining to transplanted metanephroi (Fig 3.12E), whilst PND1 sections appeared to give an increased level of staining, but still much less than that seen in adult tissue (Fig 3.12F).

3.3.2.6 Na-K-2Cl co-transporter type 2 (NKCC2)

NKCC2 was localised to the thick ascending limbs of the loop of Henle' and was visible in adult rat kidney (Fig 3.13A). This staining was not apparent when secondary antibody only was applied (Fig 3.13B). NKCC2 was also localised to the thick ascending limbs of transplanted metanephroi (Fig 3.13C), E21 (Fig 3.13D-E) and PND1 kidneys (Fig 3.13F) though staining appeared much less intense than was observed in adult rat kidney.

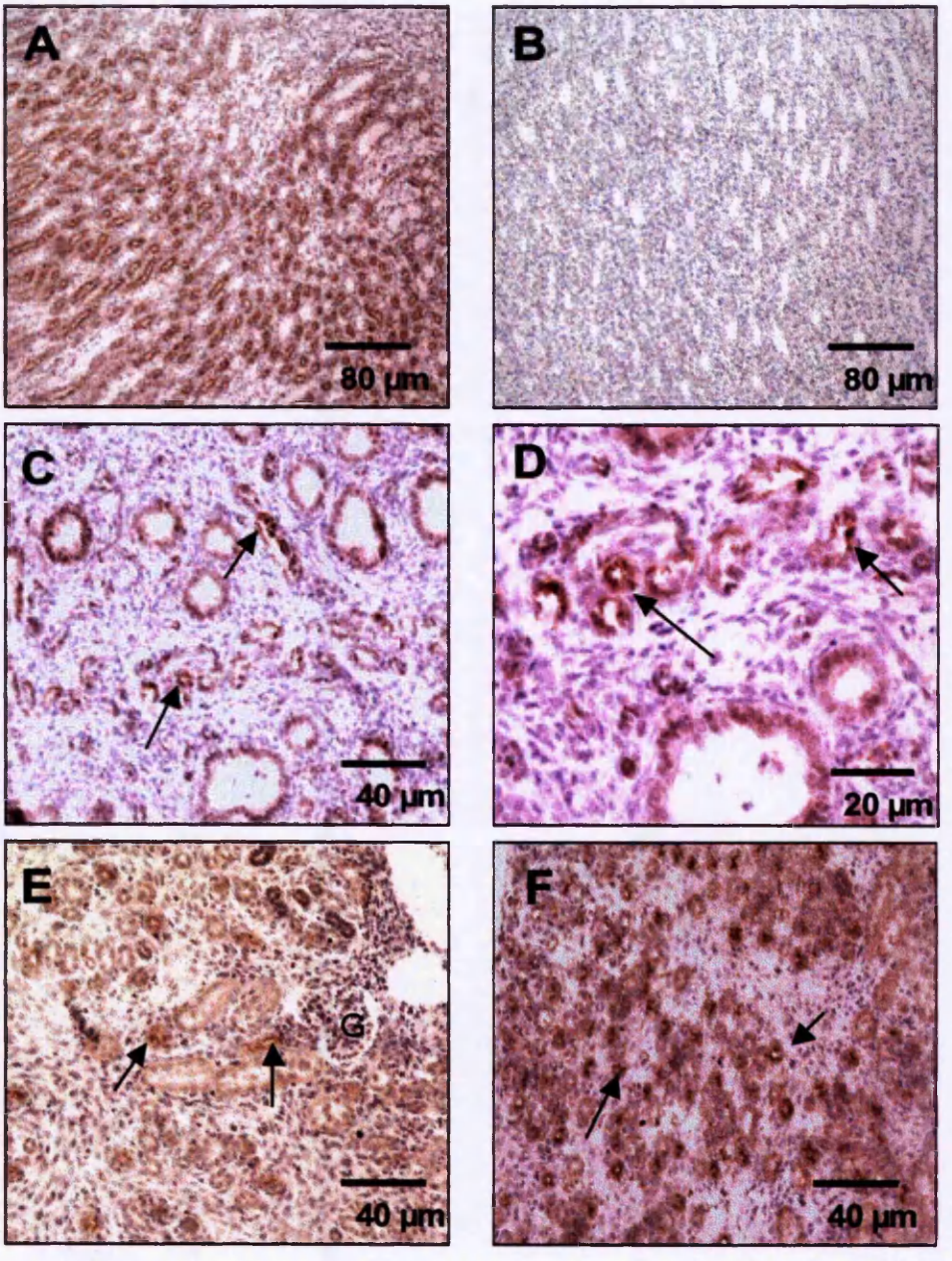

Figure 3.12. Immunohistochemical localisation of ENaC. Localised is the α-subunit of ENaC. Intense staining is shown in the collecting ducts of the outer medulla of adult rat controls (A) but not in sections (B) that had no primary antibody applied. Staining is apparent in transplanted metanephroi in collecting ducts (arrowed) in (C) and (D). Staining is also visible in the cortical collecting ducts, near to the glomeruli (G) of embryonic day 21 rats (E) and outer medullary collecting ducts of post-natal day 1 animals (F). Original magnifications for A and B are x50. C,E and F are x100 and D is x200.

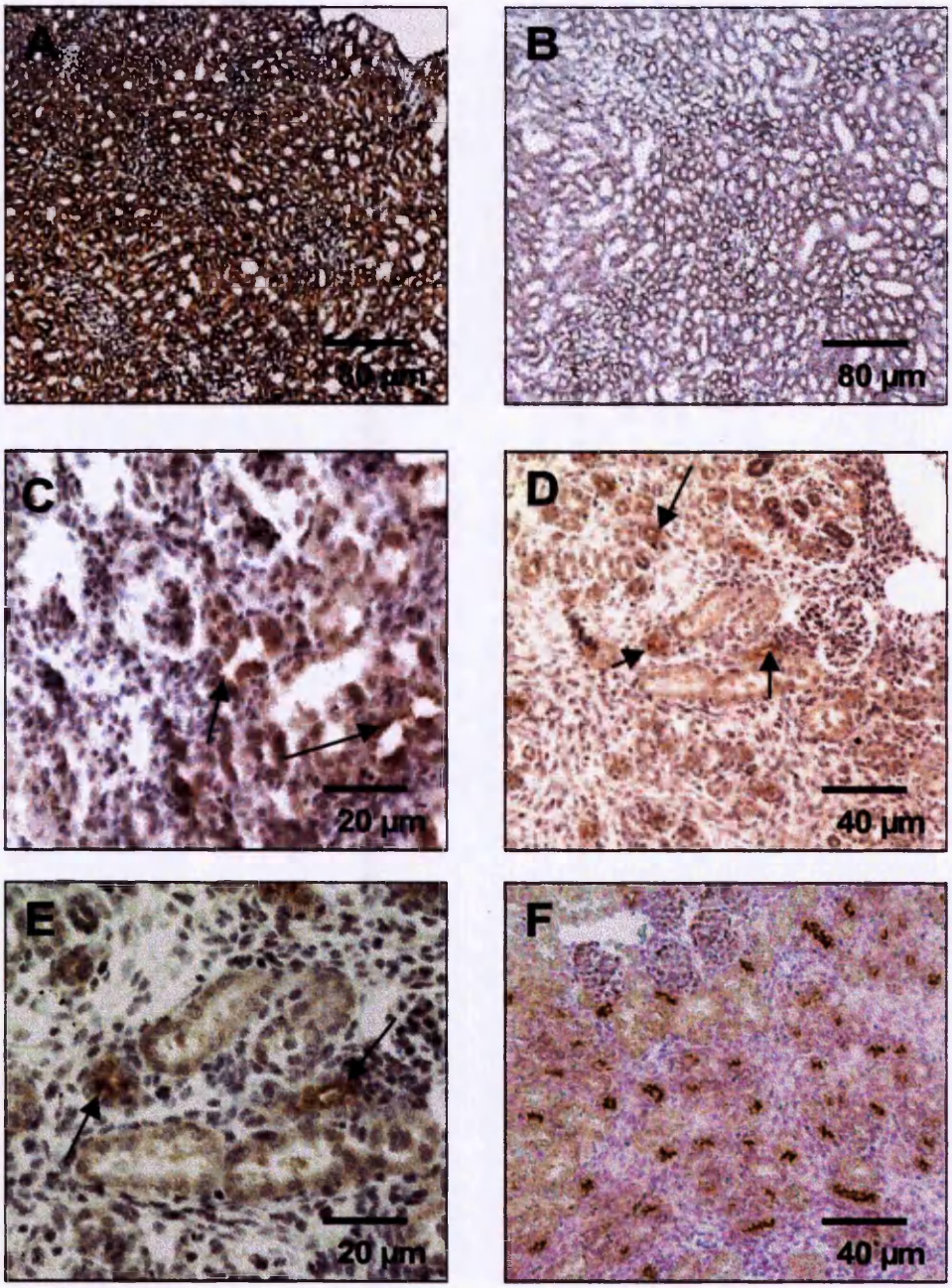

Figure 3.13. Immunohistochemical localisation of NKCC2. Intense staining is shown in the thick ascending limbs of adult rat controls (A) but not in sections (B) that had no primary antibody applied. Staining is apparent in the thick ascending limbs (arrowed) of transplanted metanephroi (C), embryonic day 21 (E21) animals (D-E) and post-natal day 1 (PND1) animals (F). Original magnifications for A and B are x50. C and E x200 and D and F x100.

3.3.2.7 Angiotensin II type 2 receptor (AT_2R)

AT_2R is highly expressed in embryonic kidney but it is only found at very low levels in adult kidney. Thus, E21 animals were used as positive controls rather than adult renal tissue. Immunohistochemistry showed localised staining in the developing tubules within the cortex of E21 kidneys (Fig 3.14A-B). This staining was not present when primary antibody only was applied (Fig 3.14C). Adult kidneys showed no visible staining for AT_2R (Fig 3.14D) in comparison with transplanted metanephroi where staining, again in the developing tubules, was evident (Fig 3.14E), though this appeared less intense than in E21 kidneys. PND1 animals showed evident staining within the renal cortex (Fig 3.14F). It must also be noted that staining for AT_2R in transplanted metanephroi was only visible in 2 out of 6 transplants examined.

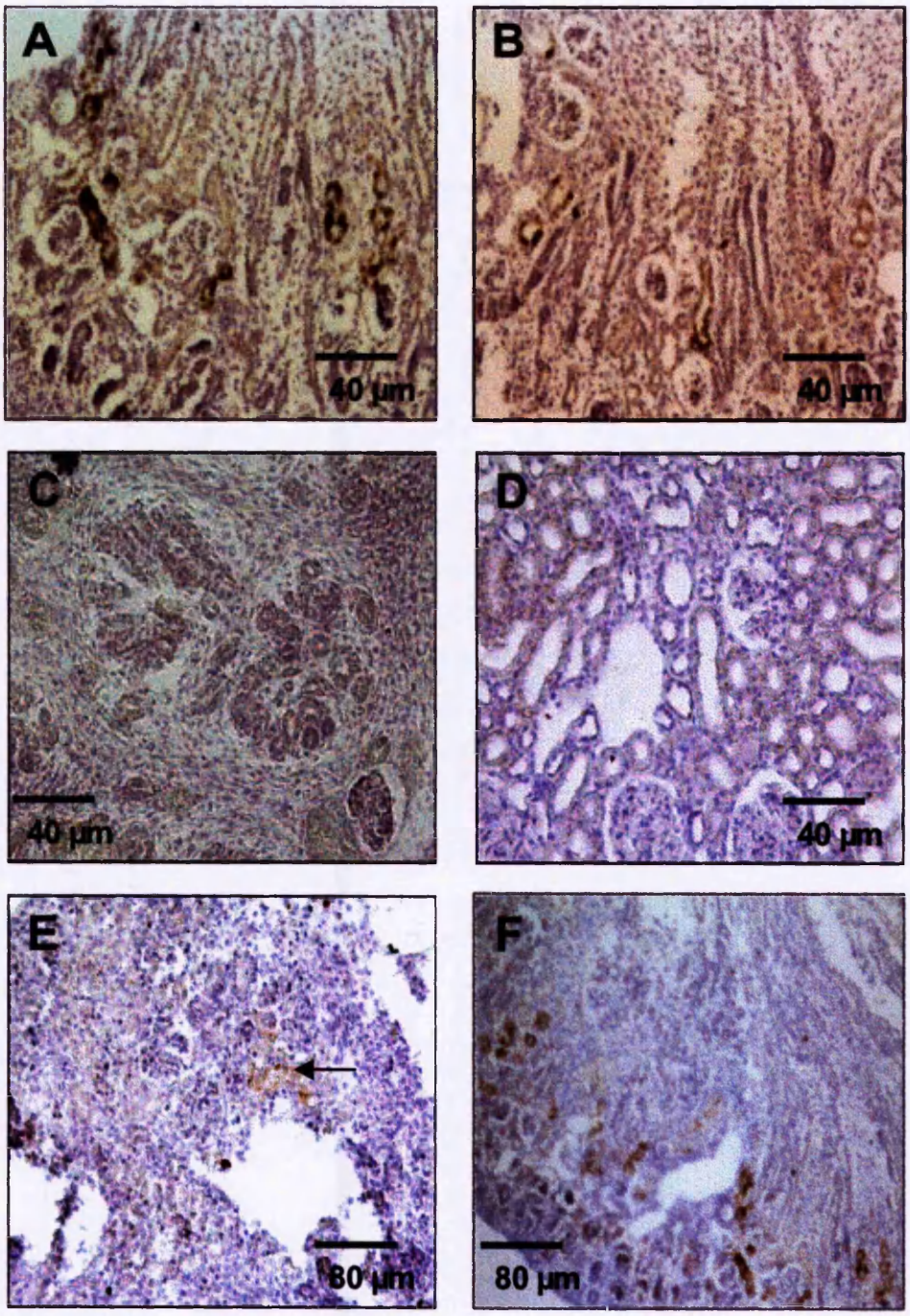

Figure 3.14. Immunohistochemical localisation of AT_2R. Intense staining is shown in the developing tubules within the cortex of embryonic day 21 (E21) control animals (A-B) but not in E21 sections © that had no secondary antibody applied. Staining is not apparent in adult rat kidney (D), but is visible in developing tubules of transplanted metanephroi (arrowed, E) and post-natal day 1 (PND1) animals (F). Original magnifications for A, B, C and D are x100. E and F are x50.

3.3.2.8 *Summary of Immunohistochemical study*

A summary of positive immunohistochemical staining in adult kidneys, transplanted metanephroi, E21 and PND1 kidneys is shown in table 3.4. The numbers indicate how many kidneys from each age showed positive staining.

	Adult kidney	Transplants	E21 kidney	PND1 kidney
AQP 1	3/3	5/6	5/5	5/5
AQP2	3/3	4/5	5/5	5/5
AT_2R	0/3	2/6	5/5	3/4
ENaC	3/3	4/5	5/5	5/5
NKCC2	3/3	4/5	5/5	5/5
UT-A1/2	3/3	0/5	0/5	0/5
UT-A1/3	3/3	0/5	0/5	0/5

Table 3.4. A summary of immunohistochemical staining. Staining within adult kidneys, transplants, embryonic day 21 (E21) and postnatal day 1 (PND1) kidneys are shown. The target proteins were as follows; aquaporins 1 and 2 (AQP1 and 2), angiotensin II type 2 receptor (AT_2R), epithelial Na channel (ENaC), Na-K-2Cl co-transporter type 2 (NKCC2) and the urea transporters A-1,2 and 3 (UT-A1,2 and 3). The numbers refer to the number of kidneys in which staining was present/total number of kidneys tested for each group.

3.3.3 Quantitative PCR

3.3.3.1 Urea transporters A1,2 and 3 (UT-A1,2 and 3)

The UT-A1 gene also encodes UT-A2 and UT-A3, which are smaller products showing significant overlap with UT-A1 itself, so it was not possible to design primers and probes specifically for UT-A1,2 or 3 separately. For this reason, mRNA expression of 2 products was quantified: UT-A1/2 combined (Fig 3.15A) and UT-A1/3 combined (Fig 3.15B). Both UT-A1/2 and UT-A1/3 had very low mRNA expression levels in transplanted metanephroi, E21 and PND1 kidneys compared to the mRNA expression within adult kidney (given an arbitrary expression value of 1). UT-A1/2 mRNA expression was not significantly different in transplanted metanephroi compared to either E21 kidneys ($P = 0.771$) or PND1 kidneys ($P = 0.056$). UT-A1/2 levels of expression were not significantly different between E21 and PND1 kidneys ($P = 0.172$). UT-A1/3 mRNA expression levels were significantly higher in PND1 kidneys compared to both transplanted metanephroi ($P = 0.001$) and E21 kidneys ($P = 0.046$). mRNA expression in the transplants was barely detectable and was significantly less than in E21 kidneys ($P = 0.001$).

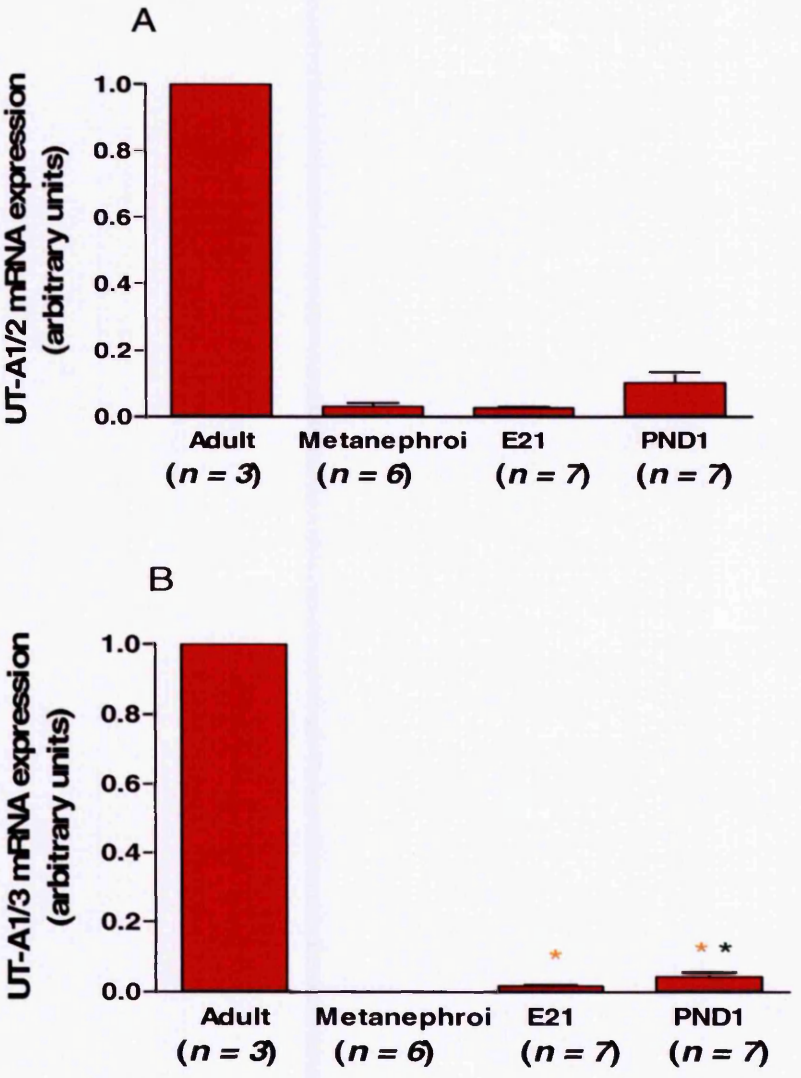

Figure 3.15. mRNA expression of UT-A1,2 and 3 by qPCR. mRNA expression of UT-A1/2 (A) and UT-A1/3 (B) in transplanted metanephroi, embryonic day 21 animals (E21) and postnatal day 1 animals (PND1). All values were compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA following log transformation of data. $P < 0.05$ was deemed significant.* $P = 0.001$ compared to transplanted metanephroi, * $P = 0.046$ compared to E21 kidneys.

3.3.3.2 Aquaporin 1 and 2 (AQP1 and 2)

mRNA expression of AQP1 (Fig 3.16A) and AQP2 (Fig 3.16B) was greatly reduced in the three experimental groups compared with adult control levels. mRNA expression of AQP1 in transplanted metanephroi was around 10% of adult levels. This however was not significantly different from either E21 ($P = 0.845$) or PND1 kidneys ($P = 0.895$). There was no statistical difference in mRNA expression of AQP1 between E21 and PND1 animals ($P = 0.897$). AQP2 mRNA expression showed a similar trend to AQP1, with no statistically significant difference in AQP2 expression in transplanted metanephroi compared to both E21 ($P = 0.243$) and PND1 kidneys ($P = 0.293$). E21 and PND1 kidneys did not differ significantly in AQP2 expression ($P = 0.997$).

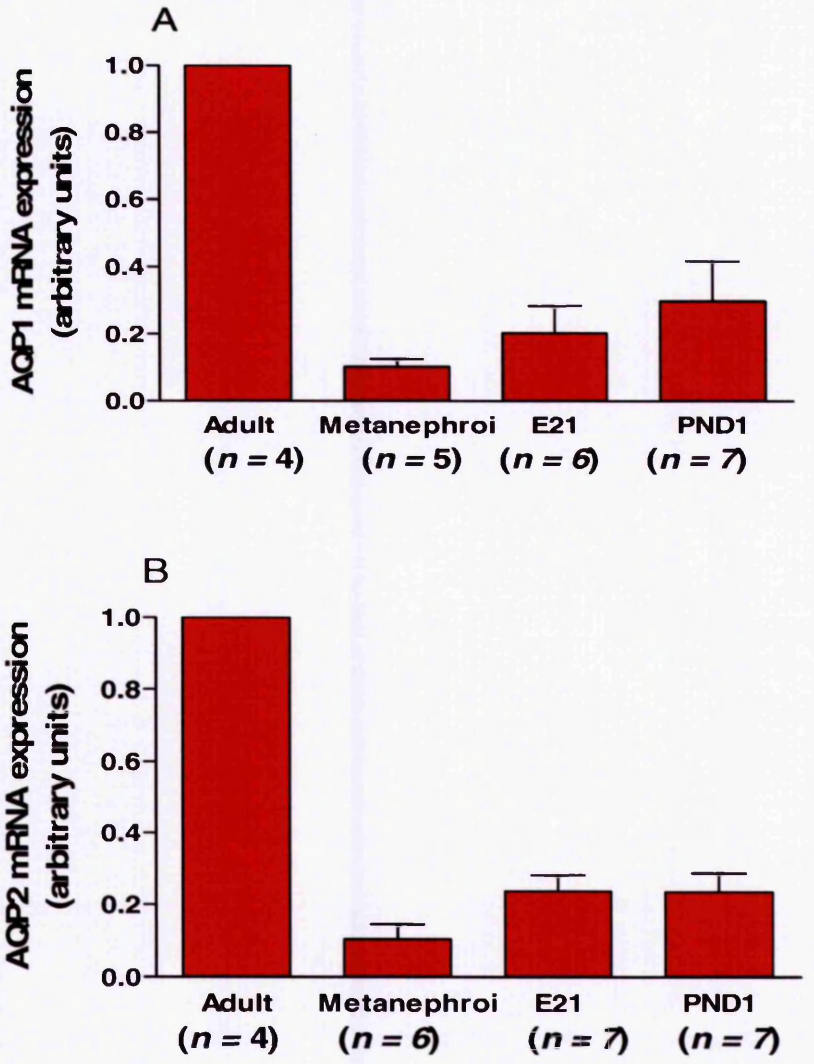

Figure 3.16. mRNA expression of AQP1 and 2 by qPCR. mRNA expression of aquaporin 1 (AQP1) (A) and aquaporin 2 (AQP 2) (B) in transplanted metanephroi, embryonic day 21 animals (E21) and postnatal day 1 animals (PND1). All values were compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA (B) and by one-way ANOVA following log transformation of data (A). $P < 0.05$ was deemed significant.

3.3.3.3 Epithelial sodium channel (ENaC)

ENaC mRNA expression was very similar in transplanted metanephroi, E21 and PND1 kidneys, at around 10% of the adult levels of expression (Fig 3.17). There was no statistical difference in the mRNA expression of

ENaC between transplanted metanephroi and either E21 (P = 0.629) or PND1 kidneys (P = 0.953). Likewise, there was no significant difference in mRNA expression between E21 and PND1 kidneys (P = 0.428).

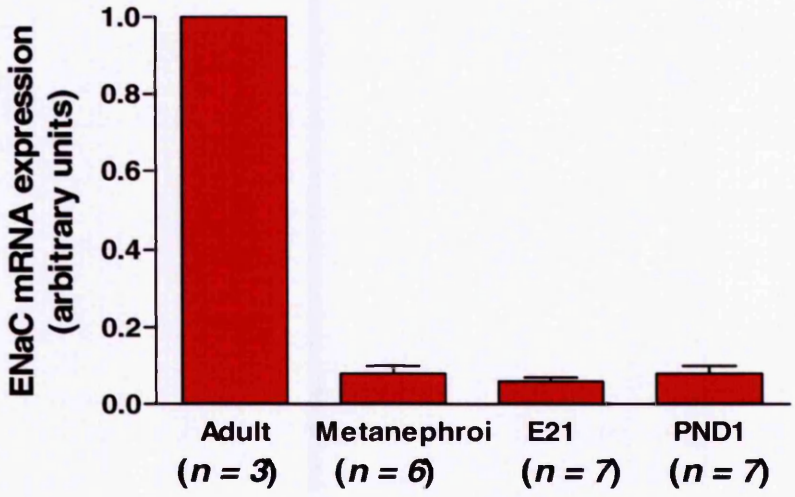

Figure 3.17. mRNA expression of ENaC by qPCR. mRNA expression of the α-subunit of the epithelial sodium channel (ENaC) in transplanted metanephroi, embryonic day 21 animals (E21) and postnatal day 1 animals (PND1). All values were compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA. $P < 0.05$ was deemed significant.

3.3.3.4 Na-K-2Cl co-transporter type 2 (NKCC2)

NKCC2 mRNA expression in transplanted metanephroi was much reduced compared with the expression within adult rat kidney (Fig 3.18). Similar to ENaC expression, there was no statistical difference in mRNA expression of NKCC2 between transplanted metanephroi and E21 kidneys (P = 0.220). The apparent difference between NKCC2 expression in the transplants and PND1 animals just failed to reach significance (P = 0.051)

whilst there was again no statistical difference between expression levels in E21 and PND1 kidneys ($P = 0.677$).

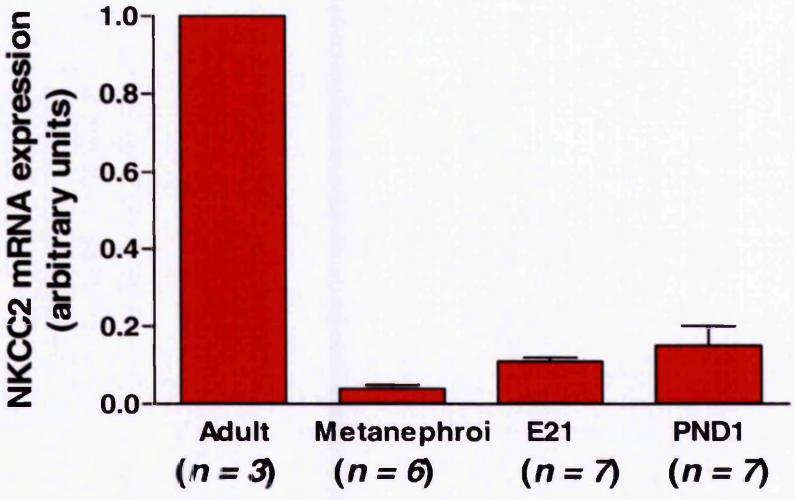

Figure 3.18. mRNA expression of NKCC2 by qPCR. mRNA expression of Na-K-2Cl co-transporter type 2 (NKCC2) in transplanted metanephroi, embryonic day 21 animals (E21) and postnatal day 1 animals (PND1). All values were compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA. $P < 0.05$ was deemed significant.

3.3.3.5 Angiotensin II type2 receptor (AT_2R)

The developmental marker, AT_2R, exhibited a completely different trend to all the other targets studied for mRNA expression in the current study (Fig 3.19). Mean mRNA expression of AT_2R in transplants, E21 and PND1 kidneys were all greater than adult levels. Whilst AT_2R expression within transplanted metanephroi appeared increased compared to adult controls, they were well below mean mRNA levels for both E21 ($P = 0.001$) and PND1 kidneys ($P = 0.001$). E21 and PND1 kidneys had fairly similar AT_2R mRNA expression, around 1000 times greater than that observed in adult

tissue. The expression levels of E21 and PND1 kidneys were not statistically different ($P = 0.991$).

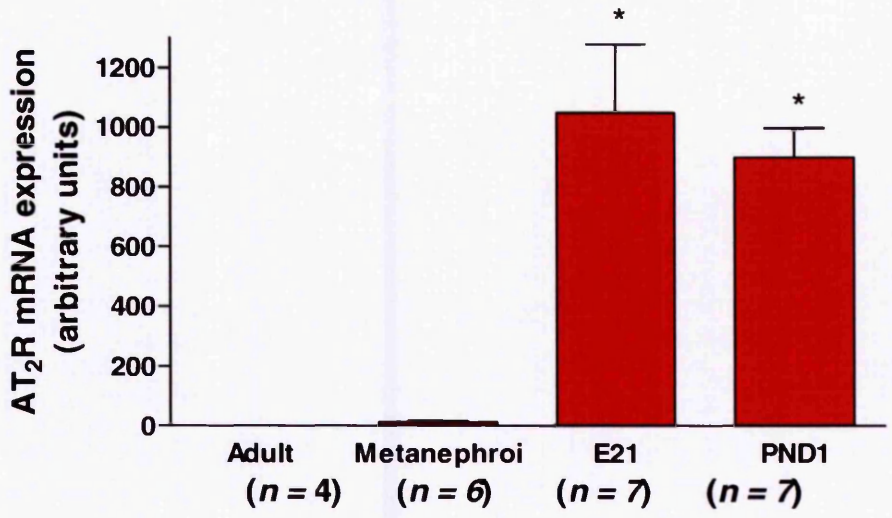

Figure 3.19. mRNA expression of AT_2R by qPCR. mRNA expression of angiotensin II type2 receptor (AT_2R) in transplanted metanephroi, embryonic day 21 animals (E21) and postnatal day 1 animals (PND1). All values were compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA following log transformation of data. $P < 0.05$ was deemed significant. * $P = 0.001$ compared with transplanted metanephroi.

3.3.4 Reverse transcriptase PCR (RT-PCR)

In all of the RT-PCR pictures that follow, there are 8 lanes per gene. These lanes were organised as follows: lanes 1-2 adult samples, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 samples. Where β-actin expression is shown (as a positive control), the same layout of lanes applied, with the exception that these lanes were numbered 9-16. Negative controls (either no cDNA or primers) were run but are not shown.

3.3.4.1 *Urea transporters A-1,2 and 3 (UT-A1,2 and 3)*

RT-PCR identified UT-A1 and 2 mRNA expression in adult rat kidneys (Figure 3.20A, lanes 1-2). There was no expression of UT-A1/2 in transplanted metanephroi or E21 kidneys but low expression in PND1 kidney (lane 8). β-actin mRNA expression appeared consistent across all groups (lanes 9-16). RT-PCR identified UT-A1/3 in only one adult rat kidney sample (Figure 3.20B, lane 2) and also low mRNA expression in one E21 (lane 6) and one PND1 (lane 8) animal. There was no expression of UT-A1/3 within transplanted metanephroi. Again, β-actin expression appeared consistent across all groups (lanes 9-16).

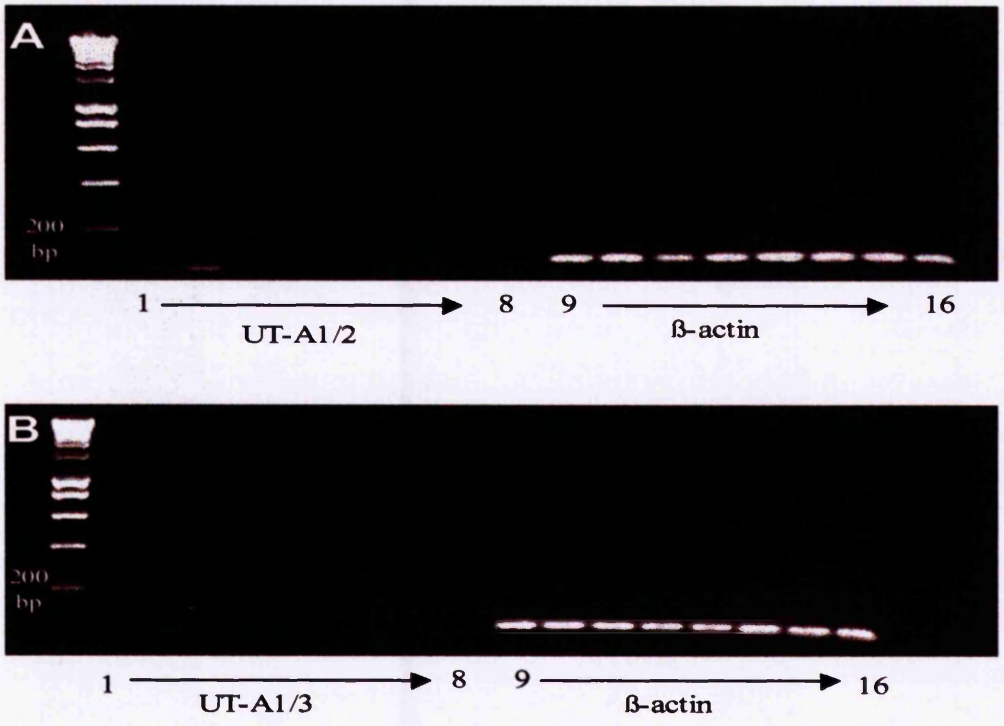

Figure 3.20. mRNA expression of UT-A1,2 and 3 by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of UT-A1/2 and β-actin (A) and UT-A1/3 and β-actin (B). Lanes 1-2 and 9-10 are adult rat kidney, lanes 3-4 and 11-12 transplanted metanephroi, lanes 5-6 and 13-14 E21 kidneys and lanes 7-8 and 15-16 PND1 kidneys.

3.3.4.2 *Aquaporins 1 and 2 (AQP1 and 2)*

RT-PCR identified AQP1 and 2 mRNA expression in all groups (Fig 3.21A and B, lanes 1-8). mRNA expression within adult kidneys and transplanted metanephroi appeared greater than E21 and PND1 animals for AQP1. AQP2 mRNA expression (Fig 3.21B, lanes 1-8) appeared higher in adult kidneys and fairly consistent for transplanted metanephroi, E21 and PND1 kidneys. There appeared to be no mRNA expression of AQP2 for one of the transplanted metanephroi (lane 4). β-actin expression was again consistent across all groups.

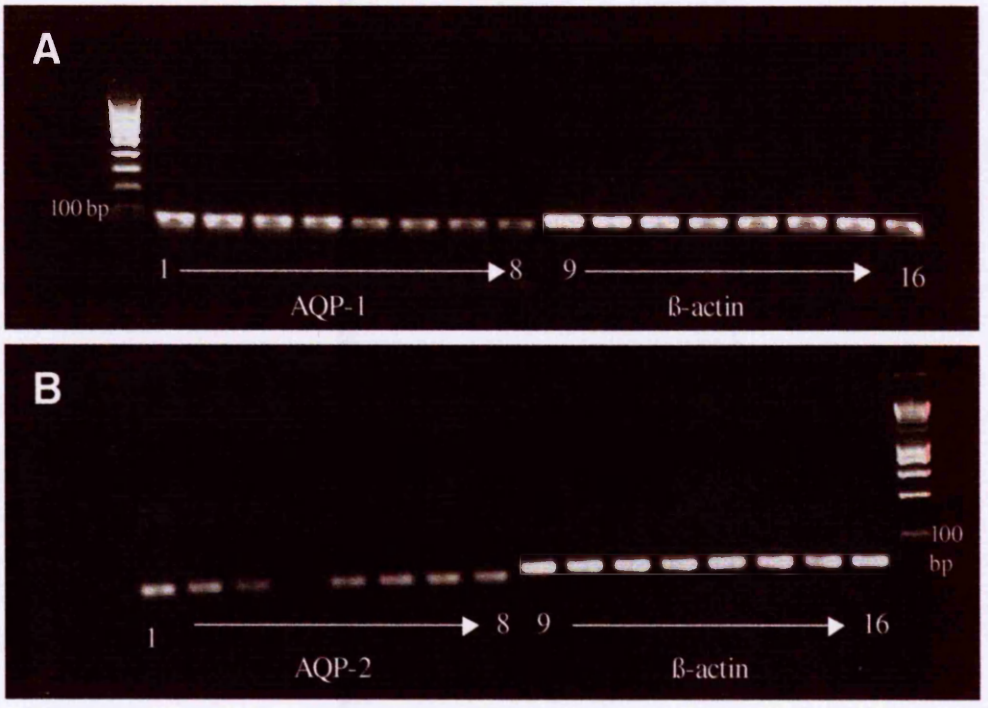

Figure 3.21. mRNA expression of AQP1 and 2 by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of AQP 1 and β-actin (A) and AQP2 and β-actin (B). Lanes 1-2 and 9-10 are adult rat kidney, lanes 3-4 and 11-12 transplanted metanephroi, lanes 5-6 and 13-14 E21 kidneys and lanes 7-8 and 15-16 PND1 kidneys.

3.3.4.3 *Epithelial Na channel (ENaC)*

Expression of ENaC mRNA, by RT-PCR (Fig 3.22) showed a higher level of expression within adult kidney (lanes 1-2) compared to transplanted metanephroi, E21 and PND1 animals. Expression in these samples was consistent except in lane 8, where one PND1 animal showed higher expression of ENaC compared with transplants, E21 and the other PND1 sample.

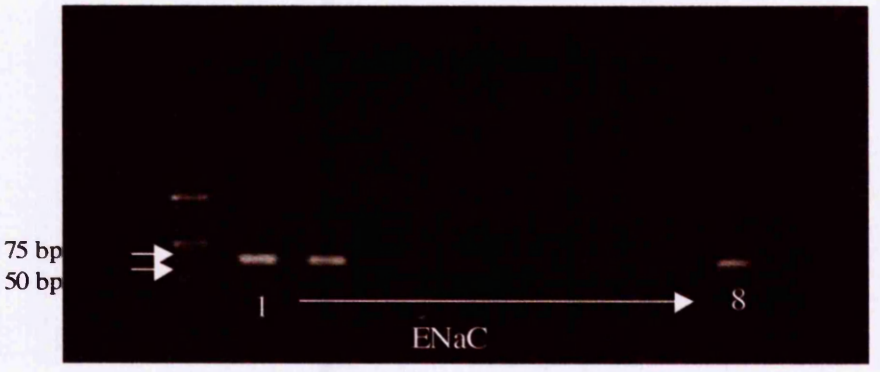

Figure 3.22. mRNA expression of ENaC by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of the α-subunit of ENaC. Lanes 1-2 are adult rat kidney, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 kidneys.

3.3.4.4 *Na-K-2Cl cotransporter type 2 (NKCC2)*

Expression of NKCC2 mRNA was fairly consistent across all groups (Fig 3.23) although there appeared to be slightly higher expression within adult and E21 kidneys (lanes 1-2 and 5-6). mRNA expression within transplanted metanephroi and PND1 animals appeared very similar.

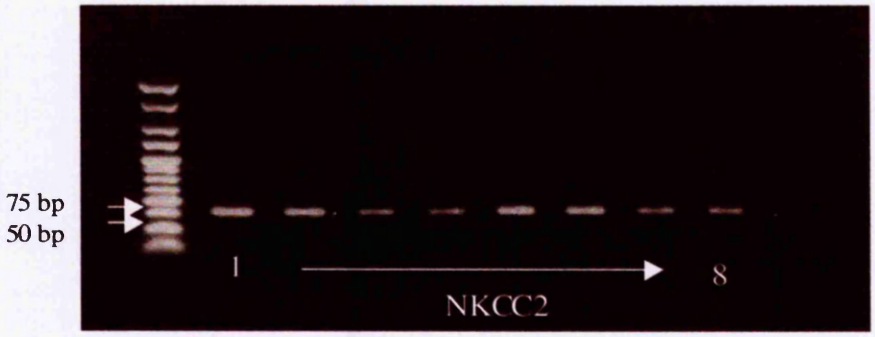

Figure 3.23. mRNA expression of NKCC2 by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of NKCC2. Lanes 1-2 are adult rat kidney, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 kidneys.

3.3.4.5 Angiotensin II type 2 receptor (AT_2R)

Expression of AT_2R mRNA, by RT-PCR was only visible in E21 and PND1 kidneys, where expression appeared fairly similar (Fig 3.24, lanes 5-8). There was a lack of mRNA expression in both adult rat kidneys and transplanted metanephroi (lanes 1-4).

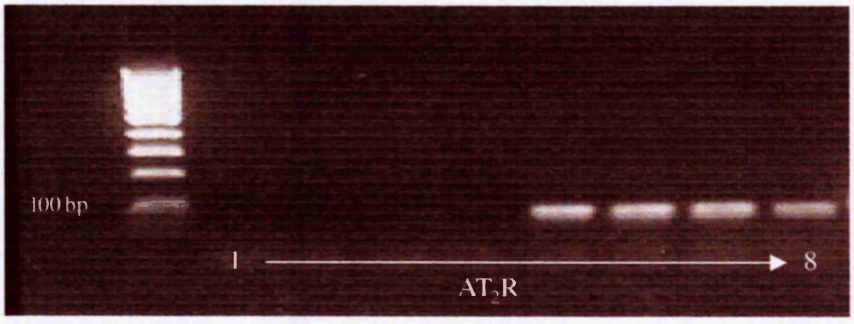

Figure 3.24. mRNA expression of AT_2R by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of AT_2R. Lanes are as follows; 1-2 adult rat kidney, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 kidneys.

3.4 Discussion

3.4.1 Glomerular counts

The estimation of glomerular number at 4200 per transplant is the first reported quantification of nephrons in transplanted metanephroi. This figure provides further useful information, as 5 of the 9 transplanted metanephroi counted were harvested at around 3 weeks post-transplant, and 4 were harvested at around 4 months post-transplant (following clearance study). Between these 2 sub-groups, there was no statistically significant difference in glomerular number. This strongly suggests that nephrogenesis within the transplants has ceased by 3 weeks following transplantation. At first glance, this may appear logical since the metanephroi, transplanted at E15, would be of an age equivalent to a kidney at postnatal day 14, by 3 weeks post-transplant. It has been shown previously that nephrogenesis in rat kidneys is complete by postnatal day 11 (Kavlock and Gray, 1982). Thus, at postnatal day 14, kidneys would be expected to have their full complement of nephrons.

However, in terms of mass, and glomerular number, the transplanted metanephroi have only grown the equivalent of 7 days. As a result, the transplanted metanephroi had significantly more nephrons than E21 kidneys, but significantly fewer than PND1 animals. Thus, up to 4 months post-transplant, transplants had a similar number of nephrons to a rat kidney around E22/postnatal day 0. Thus, despite the fact that transplanted metanephroi only possess the amount of nephrons expected

at around the time of birth in the rat, it appears that nephrogenesis has ceased within the transplants.

Nonetheless, it would appear that the transplants have the number of nephrons expected of a kidney of their size by comparison with the E21 and PND1 nephron numbers in the current study. Unfortunately, there appears to be little literature on the number of glomeruli in kidneys at around the time of birth in the rat with which to make comparisons. The only literature similar to the current study focused on absolute and relative volumes of glomeruli from E14 to E21 (Bertram et al., 2000). Seemingly, the earliest estimation of glomerular number comes from postnatal day 5 rats where it was estimated that around 19,000 glomeruli were present (Nyengaard, 1993).

Glomerular estimates in adult rats give a nephron number somewhere between 27-32,000 (Nyengaard, 1993; Woods et al., 2001a; Woods et al., 2001b; Armitage et al., 2005). Clearly, the transplanted metanephroi in the current study are limited to around 14-15% of adult nephron levels, which would severely limit the functional capacity of the transplants. It has been shown previously that adult kidneys are able to function normally with just 30% of remaining nephrons (Guyton and Hall, 2000). Thus, it may not be necessary for the transplants to possess a similar number of nephrons to adult kidneys in order for this transplantation strategy to be viable. However, the fact that these nephrons are not fully mature suggests a greater number of nephrons may be required to compensate for this immaturity.

To summarise the glomerular counts, nephrogenesis was retarded in the transplants. However, when considering that the growth of the transplants was also stunted, glomerular and thus nephron numbers appeared normal, in relation to size. Thus, a transplanted metanephros that was somewhere between an E21 and PND1 kidney in terms of size, was also between these 2 time-points in terms of nephron number. Accordingly, the size of transplanted metanephroi, and thus nephron number, will have to be improved in the future in order for a level of therapeutic function to be attained.

3.4.2 Immunohistochemical staining and quantitative and real time PCR

3.4.2.1 Urea transporters A-1,2 and 3

There are contrasting reports in the literature regarding the developmental age at which UT-A is first expressed. One group describe protein expression, by immunohistochemistry, of UT-A in the developing kidney as early as postnatal day 1 (Kim et al., 2002). It is important to note that this group examined the expression of UT-A1,2 and 4 rather than UT-A1,2 and 3 as in the current study. Since the UT-A4 isoform has yet to be localised to a specific segment of the nephron, only to the outer and inner medulla of the kidney, it could be hypothesised that the staining observed by Kim *et al* (2002) is UT-A4 since it was not possible to distinguish one UT-A isoform from another. This, however, is unlikely. A more likely reason is

that not all postnatal day 1 animals expressed UT-A but a small number did. Although not visible by immunohistochemistry, one PND1 animal in the current study gave low expression of UT-A1 and 2, identified by RT-PCR and qPCR where mRNA expression was around 10% of that of adult levels. This was also evident for UT-A1 and 3, where mRNA expression by RT-PCR was seen in one PND1 animal and also one E21 animal. This expression in E21 animals is at an earlier age than one might expect, based upon published data (Kim et al., 2002), and should be treated with caution since the mRNA expression of UT-A1 and 3 by qPCR, a much more sensitive method, is barely detectable.

Other published literature states that mRNA expression of UT-A1, by RT-PCR, was not detectable until postnatal day 14 (Liu et al., 2001). This compares well with the current immunohistochemical study whereby UT-A1 (and also UT-A2 and 3) was visible in postnatal day 14 animals (not shown) but not at earlier postnatal ages. If this is indeed accurate, it would help to explain why there was no immunohistochemical staining of UT-A1,2 or 3 in transplanted metanephroi. Size, nephrogenesis and concentrating ability of the transplants, in terms of urea, have all been shown to be consistent with a kidney at around the time of birth in the rat. If UT-A is not detectable until PND14 by immunohistochemistry, or until PND1 by PCR, then it is logical to predict that the transplants will not express the UT-A protein and have an mRNA expression that is barely detectable.

Urea transport in the kidney has been shown to be important in the formation of a concentrated urine (Hediger et al., 1996; Sands et al.,

1997). Clearly, transplanted metanephroi in the current study express UT-A transporters at low levels. This means that urea transport will be more reliant on passive diffusion, with only low levels of facilitated urea transport likely to occur. With a limited facilitated transport of urea, a fully functioning 'urinary concentrating' mechanism cannot be present. Thus, the transplants are unable to concentrate urea to adult levels and to form a concentrated urine. Despite this, urea concentration within urine produced by the metanephroi (Chapter 2) was only around 50% lower than the concentration within urine from the native kidney. Hence, the UT-A transporters (and more specifically UT-A1 and 3) that were expressed at low levels within transplanted metanephroi may have been transporting urea, particularly within the collecting duct region of the nephron.

It has been shown that newborn rats do not develop full concentrating ability until 2-3 weeks following birth (Trimble, 1970; Rane et al., 1985; Yasui et al., 1996). Thus, transplanted metanephroi in the current study, considering their size and state of nephrogenesis, would not be expected to concentrate urine to adult levels. This is an important fact as, even though transplanted metanephroi do not have a comparable concentrating ability to adult rats, they are performing at a normal and expected level of function considering their stunted growth and development. The question is, would improved growth of the transplants result in greater tubular maturity and facilitated urea transport?

3.4.2.2 Aquaporins 1 and 2

Since UT-A mRNA was only expressed at low levels in transplanted metanephroi it appeared that facilitated transport of urea was severely limited and thus the ability to concentrate of urine was significantly impaired. Hence, the 'concentration of urine' may have been largely due to the reabsorption of water. As such the aquaporins, and in particular aquaporin 1 and 2, were likely candidates for this movement of water.

Aquaporin 1 has been visualised, by immunohistochemistry, in the renal vasculature as early as E16 (Kim et al., 1999) whilst previous reports suggest that mRNA expression of aquaporin 1, although detectable in E16, becomes significant somewhere between E18 and time of birth in the rat (Smith et al., 1993; Yamamoto et al., 1997). AQP1 is highly expressed one week following birth and this expression is maintained throughout adulthood (Liu et al., 2001).

Aquaporin 2 has been detected as early as embryonic day 18 (Yamamoto et al., 1997; Baum et al., 1998) whilst it has been observed that mRNA and protein expression of AQP2 in rats does not reach maximal expression until between 4-10 weeks of age (Yamamoto et al., 1997; Bonilla-Felix, 2004).

AQP1 and 2 mRNA and protein were both detectable in E21 kidneys in the current study. This matches the previous reports for AQP2, and provides new evidence to suggest that AQP1 is actually expressed at significant levels at E21. Interestingly, the latest embryonic day measured by Yamamoto, Sasaki *et al* (1997), until time of birth, was E18. This suggests

that between E18 and E21, there is a significant increase in AQP1 expression levels.

Transplanted metanephroi did indeed express AQP1 and 2 protein and mRNA. Considering the evidence, this would appear logical based on the assumption that the transplants resemble a kidney at around the time of birth in terms of nephrogenesis and size. Since AQP1 and 2 are expressed at embryonic ages in normal metanephroi developing *in situ*, it seems likely that these channels would be expressed in transplanted metanephroi. However, this was not the case for AQP2 in one of the transplants in the RT-PCR experiment, highlighting the variability in the maturity of the transplants.

From the immunohistochemical sections, it appeared that the expression of AQP1 and 2 proteins in the transplants was less intense than in the E21 and PND1 kidneys. This trend was also apparent for the qPCR data, however this difference failed to reach statistical significance. The fact that AQP1 and 2 are present in the transplanted metanephroi suggests that water reabsorption may be occurring in various parts of the nephron, including the proximal tubules, thin descending limbs and the collecting ducts. This water reabsorption is likely to be the main method of urine 'concentration' by the transplants, particularly given the relative lack of urea transporters.

AQP1 plays a role in water reabsorption in the proximal tubules and the thin descending limb, whilst AQP2 is crucial for water reabsorption in the cells of the collecting duct. AQP1 knockout (KO) studies in mice have suggested that these animals are unable to create the hypertonic

medullary interstitium required for the countercurrent multiplication mechanism (Ma et al., 1998; Verkman, 1999). Considering that transplanted metanephroi only express AQP1 mRNA at around 10% of adult levels, it is probably fair to assume that any countercurrent multiplication will be limited, which will drastically reduce the degree to which the transplants are able to 'concentrate' urine.

It is known that an AQP2 global knock-out is lethal (Rojek et al., 2006) whilst AQP2-collecting duct KO mice (AQP2 is still expressed in the connecting tubule) are significantly smaller, had a urine output ten-times higher than wild types and a much lower urine osmolality (Rojek et al., 2006). Thus, AQP2 appears crucial in the formation of a concentrated urine and its low expression in the transplants, compared with adult controls, is a likely reason for the relatively dilute urine produced. One further point to consider is that the relatively low expression of AQP2 may have been the result of low expression of the vasopressin V_2 receptor. As previously stated, AVP is responsible for the insertion of AQP2 into the apical plasma membrane of the collecting duct (Stetson et al., 1980) and thus a lack of expression of V_2 receptor would mean less AVP binding and less shuttling (and thus expression) of AQP2 (Promeneur et al., 2000). V_2 expression in transplanted metanephroi was not measured in the current study but it is known that the V_2 receptor is expressed in rat as early as E18, increasing until it reaches adult levels (Sarmiento et al., 2005). This correlates well with the developmental expression of AQP2 (Yamamoto et al., 1997; Baum et al., 1998) and suggests that whilst the V_2 receptor would likely have been present within transplanted metanephroi, its low

expression would have led to decreased shuttling of AQP2 to the apical membrane.

3.4.2.3 Epithelial Na channel (ENaC)

To further explore the notion that water reabsorption was largely responsible for the urine concentrating mechanism within transplanted metanephroi, expression of ENaC in the transplants was assessed. ENaC mRNA is expressed as early as embryonic day 16, with expression increasing rapidly until around 3 days after birth (Vehaskari et al., 1998). ENaC was expressed in transplanted metanephroi, E21 and PND1 animals, as might be expected.

ENaC is primarily involved in sodium reabsorption by the cortical collecting ducts (Schafer, 2002) and its activity is largely controlled by aldosterone, which increases ENaC activity and increases sodium and water reabsorption within the cortical and outer medullary collecting ducts (Kemendy et al., 1992; Eaton et al., 1995; Chen et al., 1999). From the qPCR data, it can be observed that ENaC expression within transplanted metanephroi was around 5-10% of adult levels, comparable to PND1 and E21 kidneys. This low expression may have been due to a relative lack of expression of the mineralocorticoid receptor, since aldosterone binds to the mineralocorticoid receptor (MR) and activates the serum and glucocorticoid-regulated kinase (sgk) protein in order to increase ENaC activity (Bens et al., 1999; Naray-Fejes-Toth and Fejes-Toth, 2000; Shigaev et al., 2000). MR expression has only been examined as early as E19 rat kidneys, which gave a positive result. Strong expression of MR

continued right through to adulthood (Madsen et al., 2004). This suggests that MR is expressed in transplanted metanephroi although the low expression of ENaC suggests that MR expression may also be low. This low MR expression would mean that the effect of aldosterone in increasing ENaC activity is much reduced.

ENaC knockout models give further insight into the function of ENaC. A knockout of the α-subunit is lethal (Hummler et al., 1996) whilst knockout of the β-subunit (McDonald et al., 1999) led to a significant increase of sodium loss via the urine due to a reduction in sodium reabsorption by the collecting duct. This clearly demonstrates the function of ENaC in terms of sodium and water reabsorption and indicates why a reduction in ENaC expression in transplanted metanephroi, E21 and PND1 animals would lead to a less concentrated urine. This reduction in ENaC expression, combined with the reduced expression of AQP2 observed in the collecting duct, would be major contributors to the inability of the transplants to produce a concentrated urine.

3.4.2.4 Na-K-2Cl cotransporter type 2

NKCC2 is another critical transporter involved in the reabsorption of salt and water by the kidney. NKCC2 is detectable in the thin ascending limbs of rats at the time of birth (Liu et al., 2001) although expression of NKCC2 protein and mRNA were detected as early as E21 in the current study. These findings suggest that NKCC2 may also be expressed in transplanted metanephroi and PND1 animals, as was indeed the case.

The role of NKCC2 within the kidney has been extensively researched. It is known that NKCC2 plays an important role in the reabsorption of NaCl, crucial for countercurrent multiplication within the renal medulla (Gamba, 1999). A loss of function mutation of NKCC2 has been shown to cause Bartter syndrome, characterised by polyuria, high plasma renin and aldosterone concentrations, hypotension and hypokalaemic metabolic alkalosis (Bartter et al., 1962; Simon et al., 1996).

NKCC2 expression in transplanted metanephroi was only around 5-10% of adult levels, comparable with expression in E21 and PND1 animals. Low expression of NKCC2 within the transplants would likely lead to less reabsorption of NaCl in the thin ascending limb and less accumulation within the renal medulla. A reduction in the magnitude of the osmotic gradient in the medulla would in turn lead to a reduction in countercurrent multiplication. Less passive water reabsorption by the collecting duct would result in a urine that is less concentrated.

3.4.2.5 Angiotensin II type 2 receptor

AT_2R mRNA is highly expressed in embryonic rat kidneys from embryonic day 14 to postnatal day 7. There are conflicting reports beyond this time-point with some reports suggesting that AT_2R becomes undetectable by postnatal day 28 (Norwood et al., 1997) whilst other reports state that it is detectable in the adult kidney (Cao et al., 2000). Due to the fact that AT_2R is expressed more highly at embryonic ages, it has been suggested to play a role in renal morphogenesis and the function of the fetal kidney

(Robillard et al., 1995; Norwood et al., 2000). This idea was supported by the detection of AT_2R with *in situ* hybridisation, in the undifferentiated nephrogenic mesenchyme but not in immature or mature tubules or glomeruli (Shanmugam et al., 1995).

As expected, AT_2R was detected, by immunohistochemistry and RT-PCR, in E21 and PND1 animals. However, no AT_2R protein or mRNA was detected within adult kidneys, supporting the work of Norwood, Craig *et al,* (1997). There was no expression of AT_2R mRNA, by RT-PCR, within transplanted metanephroi and some expression of the AT_2R protein in 2 out of the 6 transplants tested. Once again, this shows that there is a degree of variability in the state of nephrogenesis and tubular maturity between transplants. However, the lack of AT_2R expression in the majority of transplants suggests that nephrogenesis had ceased, despite the relative immaturity of the metanephroi. The qPCR data provide further evidence for this hypothesis. It was apparent that the expression of AT_2R in the transplants was closer to adult levels of expression (barely detectable) than to the high levels of expression visible in E21 and PND1 animals.

AT_2R has recently been linked to an apoptotic role in the development of the metanephros (Wolf, 2002; Wolf, 2005), and in adult rats following injury (Ruiz-Ortega et al., 2003; Tejera et al., 2004), possibly to counter-balance the proliferative effects of the angiotensin II type 1 receptor (AT_1R) (Cao et al., 2000). The exact nature of this apoptotic role is unclear. Clearly, the lack of AT_2R in the transplants would suggest that this apoptotic role, as part of the developmental process, had ceased. Thus, it

would appear that, between 3 weeks to 4 months post-transplant, the transplanted metanephroi have developed their maximal number of nephrons.

3.4.3 Conclusions of this study

Clearly, as alluded to in the previous chapter, transplanted rat metanephroi do not grow at the same rate as a normal developing rat kidney. It was suggested in chapter 2 that the transplants, between 3 weeks to 4 months post-transplant, had only grown the equivalent of 7 days. Further evidence in support of this suggestion comes from the observation that glomerular numbers in the transplants were comparable with a rat kidney at around the time of birth. Likewise, the expression of key transporters and channels by transplanted metanephroi, essential for urinary concentration, was comparable with both E21 and PND1 kidneys, with the exception of UT-A1/3. These results suggest that, despite their chronological age, the transplants are at a stage of nephrogenesis and tubular maturity expected of a kidney of similar size.

The data presented in Chapter 2 suggested that an immature reabsorption mechanism was present in transplanted metanephroi. The urine:plasma inulin ratio was greater than unity and the urea concentration of metanephric urine was greater than that of serum. Facilitated urea transport contributes to the concentration of urea in the urine by the mature kidney. Clearly, this is limited within the tubules of the transplants,

given the low UT-A expression. Hence, water reabsorption must be taking place in order to increase urea concentration. Expression, albeit limited, of AQP1 and 2, ENaC and NKCC2 supports this suggestion. The selective re-absorption of salt and water, by the metanephric nephrons, would lead to the concentration of urea in the urine.

This putative re-absorption of water, whilst not yet able to concentrate urine to adult levels, appears to be normal for a kidney of the transplant's size. In fact, the osmolality of urine from animals surviving on transplanted metanephroi alone was greater than that of normal plasma osmolality values (around 300 mOsm/kg) (Marshall et al., 2006 *In press*). Furthermore, the urine osmolality was comparable with that reported in neonatal animals (Gray and Kavlock, 1991).

In summary, this chapter has shown that, whilst the transplants have only grown the equivalent of 7 days, the expression of key transporters and channels, and the state of nephrogenesis, appear 'normal' for a kidney of this size. Unfortunately, growth and tubular maturation at this stage of development appear to have ceased. This begs the question whether an improvement in the growth of the transplants would lead to a subsequent increase in nephrogenesis and an improved tubular concentrating ability? Clearly, the next challenge is to improve the growth of these transplants in order to improve functional capacity and advance this technology towards a therapeutic level of function. The next chapter will focus on an attempt to improve the growth of transplanted metanephroi, by improving arteriogenesis.

Chapter 4: Promotion of arteriogenesis in transplanted metanephroi

4.1 Introduction

4.1.1 Background

One of the conclusions of Chapter 2 was that in order to improve the growth and tubular maturity of transplanted metanephroi, the blood supply to the transplants needed to be improved. By analysing the renal vasculature supplying the transplants, it was apparent that smaller arteriolar vessels were being formed, probably via angiogenesis, but it appeared that these were not developing into larger conductance arteries. Arteriogenesis is described as 'the transformation of pre-existing collateral arteriolar pathways into large conductance arteries, via the recruitment of smooth muscle cells' (Carmeliet, 2000; Van Royen et al., 2001; Schaper and Scholz, 2003) and so is a separate process from angiogenesis (blood vessel formation).

The onset of arteriogenesis appears to be related to increased shear forces on the vessel wall. These shear forces arise as a result of increased flow in the collateral vessels due to an increase in the pressure gradient, often caused by a major artery occlusion. It has been suggested that vessel growth by arteriogenesis is induced by this increase in shear stress. These shear forces activate quiescent endothelial cells and the release of factors such as transforming growth factor β (TGF-β) and monocyte chemoattractant protein-1 (MCP-1) (Shyy et al., 1994; Ohno et al., 1995). Increased shear forces also upregulate the endothelial receptors for circulating monocytes (Scholz et al., 2000). The final stages

of arteriogenesis involve the docking of monocytes to the endothelium and their accumulation in the form of macrophages (Arras et al., 1998). Macrophages produce many factors believed to be important for arteriogenesis, as is described later.

Angiogenesis is defined as the sprouting of endothelial cells, leading to the formation of new capillary networks (Risau, 1997). The driving force for angiogenesis is ischaemia and hypoxia. Hypoxia causes an increase in the expression of vascular endothelial growth factors (VEGFs) leading to the increased proliferation of endothelial cells (Shima et al., 1995).

Arteriogenesis has been studied primarily in animal models of injury, where arteriogenesis was assessed on collateral arteries formed following an occlusion of one of the femoral arteries in rabbits (Buschmann et al., 2001; Hershey et al., 2001; Hoefer et al., 2001; Schirmer et al., 2004). These studies showed that the collateral arteries that formed underwent vessel enlargement, largely due to the regrowth of smooth muscle cells, up to 20-times their original size (Schaper and Ito, 1996). Although these studies were undertaken on hindlimbs, it is thought that the mechanisms of arteriogenesis are similar in different vascular beds. In the renal arterial tree, arteriogenesis may also be aided by the renin-angiotensin system, as renin has been linked to the branching of the renal vasculature (Reddi et al., 1998; Carmeliet, 2000).

Prior to making a decision about how arteriogenesis could be improved in the transplanted metanephroi, it was necessary to identify some candidate molecules that were linked to the recruitment of monocytes/macrophages

and, ultimately, arteriogenesis. Both granular monocyte colony stimulating factor (GM-CSF) and MCP-1 were shown in rabbit hindlimb studies to be pro-arteriogenic, albeit on collateral arteries derived as a result of injury (Buschmann et al., 2001; Hershey et al., 2001; Hoefer et al., 2001; Schirmer et al., 2004). MCP-1 is a chemokine and was the first substance shown to increase arteriogenesis, largely by increasing the number of circulating monocytes/macrophages (Ito et al., 1997). The exact role of monocytes in arteriogenesis is still unknown but it is believed that they play a role in the secretion of chemokines and growth factors such as transforming growth factor-β (TGF- β), the VEGFs and basic fibroblast growth factor (bFGF) (Polverini et al., 1977; Wahl et al., 1987; Berse et al., 1992; Arras et al., 1998). These factors, together with metalloproteinases produced by the monocytes (Malik et al., 1996) are believed to help create an inflammatory environment that is necessary for arteriogenesis.

GM-CSF has been shown to enhance the survival and proliferation of haematopoietic cells (Just et al., 1993; Denecker et al., 1997) and, like MCP-1, also plays a role in the recruitment of monocytes/macrophages and an amplification in their function (Buschmann et al., 2001). Consequently, it was hypothesised that dosing the transplants with GM-CSF and MCP-1, prior to transplantation, would aid arteriogenesis and the formation of larger conductance arteries within transplanted metanephroi. However, because GM-CSF and MCP-1 recruit macrophages, which play a role in the chronic rejection of transplants (Land et al., 1971; Hancock et al., 1993; Heemann et al., 1993; Beckmann et al., 2006), it was necessary to dose the transplants at relatively low doses of MCP-1 (1 μg/mL) and

GM-CSF (1 ng/mL), even in this syngeneic model of transplantation. These doses chosen were 10-times the EC_{50} value, which was similar to the doses chosen for the 12-growth-factor cocktail used to stimulate overall growth of the metanephroi.

Prior to initiating a study to assess whether GM-CSF and MCP-1 could improve arteriogenesis, it was first necessary to assess the expression of endothelial and smooth muscle markers in the transplants. The endothelial markers chosen were rat endothelial cell antigen-1 (RECA-1) and the platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31). RECA-1 is an endothelial marker specific to rats and stains all endothelium within kidney, including glomeruli, vasa recta and arterial and capillary networks (Ricono et al., 2003; Takeda et al., 2006). CD31 is expressed on the surface of platelets and certain lymphocyte subsets (Watt et al., 1995) and also on the plasma membrane of endothelial cells (Scholz and Schaper, 1997) and is used as a marker of vascular development (Baldwin et al., 1994). The vascular smooth muscle marker employed was vascular α-smooth muscle actin (v α-SMA). v α-SMA is abundantly stained, by immunohistochemistry, in the vascular smooth muscle of the developing and adult kidney (Carey et al., 1992; Park et al., 1997; Ranieri et al., 2001).

4.1.2 Aims of the study

The first aim of this part of the study was to assess the expression of RECA-1, CD31 and v α-SMA within transplanted metanephroi. As the

nucleotide sequence of RECA-1 is not yet known, RECA-1 was examined by protein expression only, using immunohistochemistry. CD31 and v α-SMA were examined for protein expression by immunohistochemistry, and for mRNA expression by RT-PCR and qPCR. In order to obtain an idea of relative expression of RECA-1, CD31 and v α-SMA in transplanted metanephroi, E21, PND1 and adult kidneys were also examined for expression of these markers.

The second part of this study focused on the theme of arteriogenesis. To investigate whether dosing the transplants with GM-CSF and MCP-1 (experimental group) improved arteriogenesis, mRNA expression of CD31 and v α-SMA was examined by qPCR and compared with transplants that had not been dosed with GM-CSF and MCP-1 prior to transplantation (control group). In order to further assess whether there was any difference in tubular maturity between experimental and control groups, mRNA expression of aquaporin 1 and 2 (AQP1 and 2), urea transporters A-1,2 and 3 (UT-A1, 2 and 3), sodium-potassium-2chloride cotransporter type 2 (NKCC2), epithelial sodium channel (ENaC) and angiotensin II type 2 receptor (AT_2R) were all examined by qPCR.

4.2 Materials and Methods

4.2.1 Animals

The animals employed in this study were Lewis rats (Charles River, UK) which were housed in the Biological Services Unit (BSU) at the University of Manchester. Animals were maintained in a 12-hour light/dark cycle with free access to water and food (Bantin and Kingman rat and mouse expanded diet, Hull, UK).

4.2.2 Transplantation of metanephroi

Transplantation of metanephroi was identical to the method described in 2.2.3.1 with the following exceptions: once E15 metanephroi had been dissected, one group of transplants (n = 6) were incubated in ice-cold DMEM media with the 12 growth-factor cocktail described previously (control group). A second group of transplants (n = 6) were incubated in ice-cold DMEM with the 12 growth-factor cocktail supplemented with 1 µg/mL MCP-1 (Sigma-Aldrich, UK) and 1 ng/mL GM-CSF (Sigma-Aldrich, UK) (experimental group). The 6 transplants from both the control group and experimental group were transplanted adjacent to the aorta in female Lewis rats. Thus, one animal had 6 control transplants and one animal had 6 experimental transplants. Animals were not dosed with methylprednisolone in this experiment as the results in Chapter 2 showed

that growth of the transplants was not improved by dosing with methylprednisolone, and that weights of metanephroi were comparable to others (Rogers et al., 1998; Rogers et al., 2001).

4.2.3 Explantation of transplanted metanephroi

Three weeks following transplantation, host Lewis rats were anaesthetised with Isoflurane (flow rate 1 L/min O_2, 2.5% Isoflurane) and a midline laparotomy was performed. Each transplanted metanephros, from experimental and control groups, was dissected free of surrounding tissue and weighed before being snap frozen quickly in liquid nitrogen (BOC group, UK). All samples were stored at –80 °C until required. Host rats were humanely euthanased by schedule 1 methods (Home Office, UK) following dissection of transplanted metanephroi

All transplanted metanephroi, E21, PND1 and adult kidneys used for immunohistochemistry and PCR experiments to measure baseline expression of CD31 and v α-SMA were explanted as described in Chapters 2 and 3.

4.2.4 Immunohistochemical staining

All tissue was embedded, sectioned and processed for immunohistochemical staining as described in Chapter 3 with the exception that different primary and secondary antibodies were used, as shown in table 4.1. All primary antibodies were raised in mouse and the

secondary antibody was a goat-anti mouse immunoglobulin labelled with horseradish peroxidase (Dako Ltd, Cambridge) used at 1:100. The specificity of these antibodies shown below has been shown previously (Skalli et al., 1986; Takeda et al., 2006).

Target protein	Dilution used	Supplier
CD31	1:100	Serotec
RECA-1	1:100	Serotec
v α-SMA	1:500	Sigma-Aldrich

Table 4.1. Antibodies employed in immunohistochemical studies. Target proteins are platelet endothelial cell adhesion molecule 1 (CD31), rat endothelial cell antigen (RECA-1) and vascular α-smooth muscle actin (v α-SMA). All primary antibodies were raised in mouse.

4.2.5 mRNA expression of target genes

Methods for total RNA extraction, cDNA conversion and RT-PCR and qPCR experiments were identical in this study to those described in Chapter 2. This included the methods used to calculate gene efficiency and relative mRNA expression and the primers and probes used for the reference gene, β-actin. The only differences were the primers and Taqman probes used, which are shown in table 4.2, and the optimum concentrations at which the primers and probes were used (table 4.3).

CD31	Forward primer 5' AGCCCCGGTGGATGAAGT 3' Reverse primer 5' CACCGAAGCACCATTTCATCT 3' Taqman probe 5' ACGATCTCCATCCTGTCGGGTAACGAT 3'
v α-SMA	Forward primer 5' CACGGCATCATCACCAACTG 3' Reverse primer 5' CCACGCGAAGCTCGTTATAGA 3' Taqman probe 5' ACGACATGGAAAAGATCTGGCACCACTC 3'

Table 4.2. Primer and Taqman probe sequences used for qPCR.

Target gene	Forward primer (nM)	Reverse primer (nM)	Taqman probe (nM)
CD31	300	300	150
v α-SMA	300	900	150

Table 4.3. Optimum concentrations of primers and probes for qPCR.

4.2.6 Statistical analysis

Data are presented as the mean ± SEM. All statistical analyses were carried out using the Statistical Package for Social Sciences version 13.0 (SPSS 13.0 for Windows). Statistical analysis was by one-way ANOVA for the qPCR data comparing mRNA expression of CD31 and v α-SMA between transplanted metanephroi, E21 and PND1 kidneys. Statistical analysis in the study to investigate promotion of arteriogenesis was by independent samples t-test. Statistical significance was set at the 5 % level.

4.3 Results

4.3.1 Immunohistochemical localisation of endothelium and smooth muscle

The transplanted metanephroi described in sections 4.3.1 and 4.3.2 were explanted following clearance study, around 4 months post-transplant, as in Chapter 3. For all of the following target proteins, 2 negative control samples were used, one slide with primary antibody only and one with secondary antibody only. However, only one of these controls is shown for each target protein in the figures that follow.

4.3.1.1 Rat endothelial cell antigen-1 (RECA-1)

RECA-1 was highly expressed in adult rat kidney (Fig 4.1A), particularly within the renal papilla and along the vasa recta. This staining was no longer apparent when only the primary antibody was applied (Fig 4.1B). Staining was prominent in the vasculature of transplanted metanephroi (Fig 4.1C-D), specifically in the peritubular capillary network. E21 and PND1 kidneys (Fig 4.1E-F) also showed staining for RECA-1 within the vasculature, though this appeared slightly less intense than in the transplants.

4.3.1.2 *Platelet endothelial cell adhesion molecule-1 (CD31)*

CD31 was also highly expressed in adult rat kidney, in similar regions to RECA-1 and also within the glomeruli (Fig 4.2A-B). This staining of CD31 was not visible when only the secondary antibody was applied to the section (Fig 4.2C). CD31 protein was localised within transplanted metanephroi (Fig 4.2D), E21 (Fig 4.2E) and PND1 (Fig4.2F) kidneys, though this staining appeared less intense than in adult kidney.

4.3.1.3 *Vascular α-smooth muscle actin*

Immunohistochemistry identified intense v α-SMA staining within the vasculature of adult kidneys, including the descending vasa recta (Fig 4.3A). Following incubation with secondary antibody alone (Fig 4.3B) this localised staining was no longer observed. The expression of v α-SMA protein was also intense within transplanted metanephroi (Fig 4.3C-D), E21 (fig 4.3E) and PND1 (Fig 4.3F) kidneys. Most of the v α-SMA staining which occurred in these groups was localised in the vasculature surrounding the tubules and glomeruli.

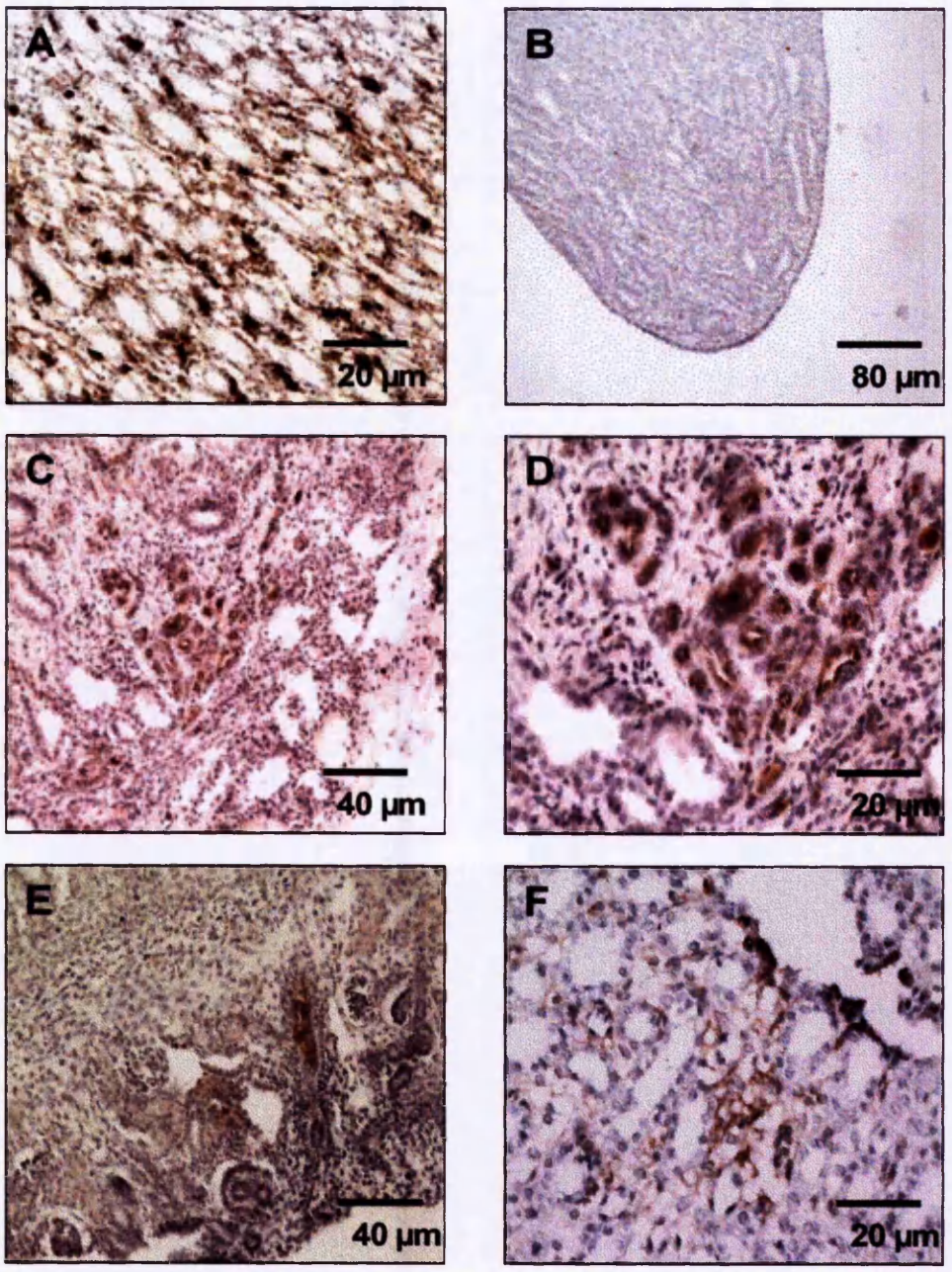

Figure 4.1. Immunohistochemical localisation of RECA-1. Intense staining is shown in the papilla of adult rat controls (A) but not in control sections that had no secondary antibody applied (B). Staining is apparent in the vasculature surrounding the tubules in transplanted metanephroi (C-D). Staining is also visible in the vasculature of embryonic day 21 (E) and post-natal day 1 animals (F). Magnifications for A, D and F are x200. B is x50 and C and E are x100.

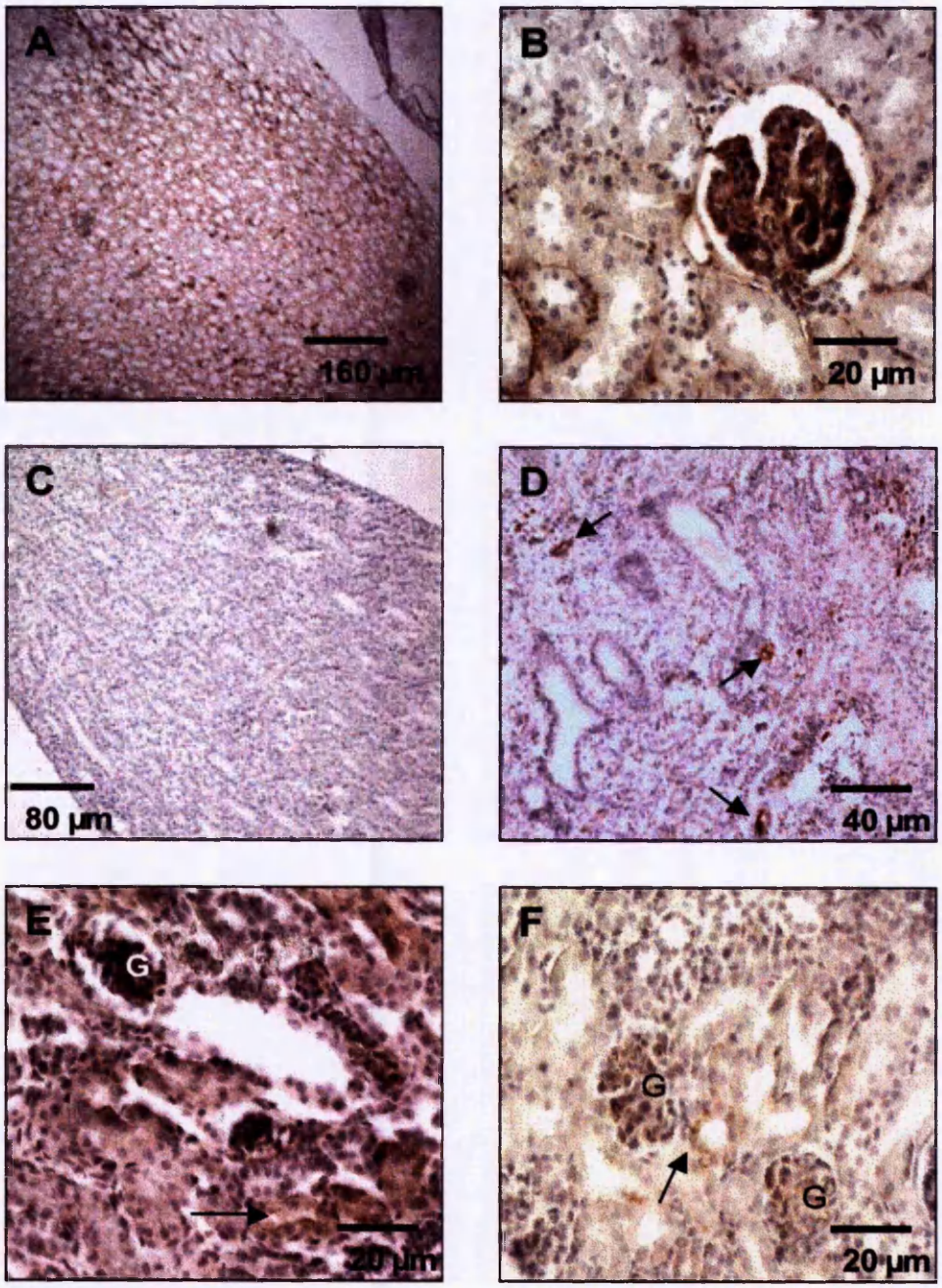

Figure 4.2. Immunohistochemical localisation of PECAM-1. Intense staining is shown in the papilla (A) and glomerulus (B) of adult rat controls but not in control sections that had no primary antibody applied (C). Staining is observed in the vasculature surrounding the tubules (arrowed) in transplanted metanephroi (D). Staining is apparent in the glomeruli (G) and vasculature surrounding the tubules (arrowed) in embryonic day 21 (E) and post-natal day 1 animals (F). Magnification for A is x25, B, E and F are x200. C is x50 and D is x100.

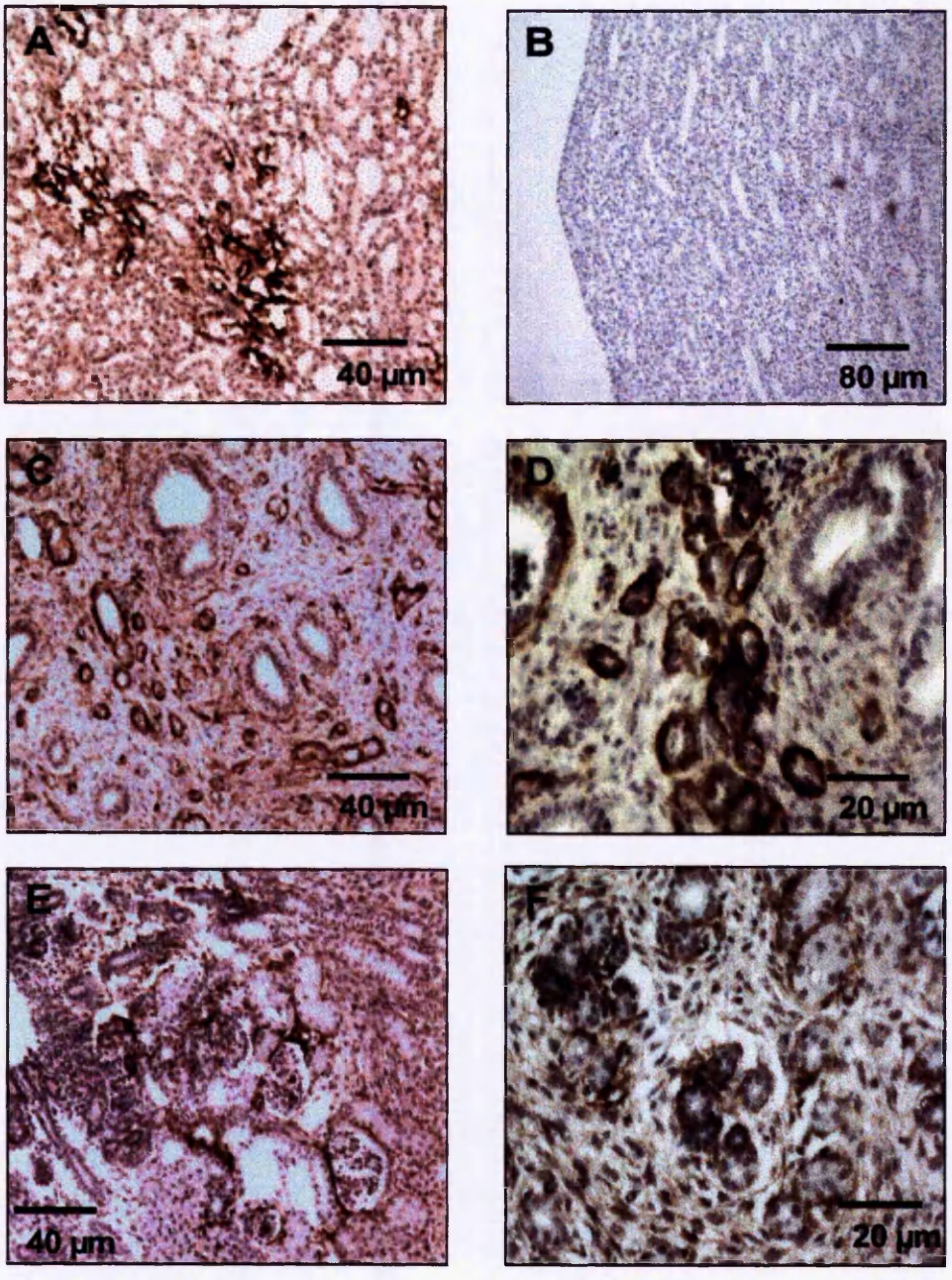

Figure 4.3. Immunohistochemical localisation of v-αSMA. Intense staining is shown in the descending vasa recta of adult rat controls (A) but not in control sections that had no primary antibody applied (B). Staining is observed in the vasculature in transplanted metanephroi (C-D) and is also apparent in embryonic day 21 (E) and post-natal day 1 animals (F). Magnification for A, C and E is x100, B is x50. D and F are x200.

4.3.2 mRNA expression of endothelium and smooth muscle

As stated previously, because RECA-1 has yet to be sequenced, only CD31 and v α-SMA mRNA expression were investigated.

4.3.2.1 qPCR of CD31 and v α-SMA

CD31 mRNA expression (Fig 4.4) within transplanted metanephroi appeared comparable with adult expression levels and higher than E21 and PND1 animals. Statistically however, there was no significant difference between transplanted metanephroi and both E21 (P = 0.548) and PND1 (P = 0.104) animals. Neither was there a significant difference for mRNA expression of CD31 between E21 and PND1 kidneys (P = 0.420).

mRNA expression of v α-SMA (Fig 4.5) was comparable between all groups, including adult kidneys. There was no statistical difference in mRNA expression between transplanted metanephroi and either E21 (P = 0.987) or PND1 (P = 0.917) kidneys. One-way ANOVA also failed to detect any difference between E21 and PND1 kidneys (P = 0.845).

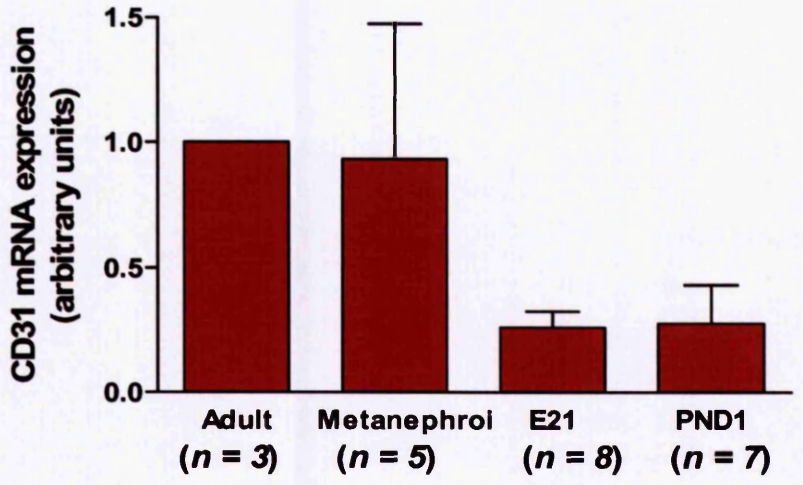

Figure 4.4. mRNA expression of PECAM-1 by qPCR. mRNA expression of platelet endothelial cell adhesion molecule-1 (CD31) in transplanted metanephroi, embryonic day 21 animals (E21) and post-natal day 1 animals (PND1). All values are expressed as mean ± S.E.M and compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant.

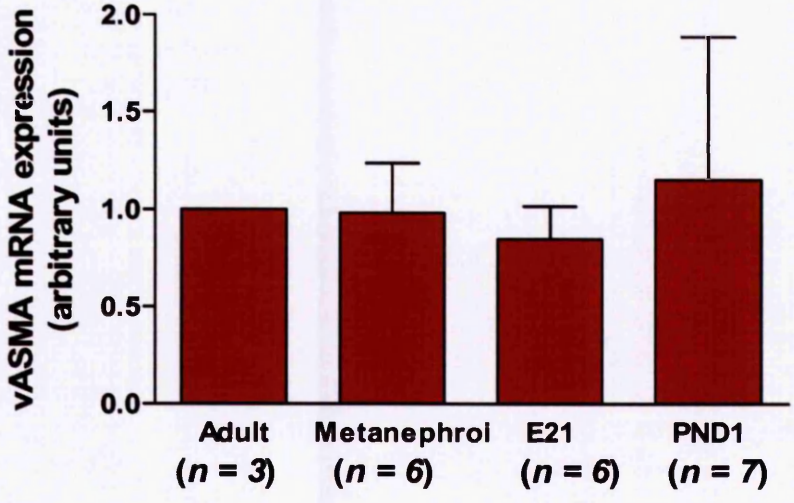

Figure 4.5. mRNA expression of v-αSMA by qPCR. mRNA expression of vascular α-smooth muscle actin (v α-SMA) in transplanted metanephroi, embryonic day 21 animals (E21) and post-natal day 1 animals (PND1). All values are expressed as mean ± S.E.M and compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by one-way ANOVA following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant.

In all of the RT-PCR pictures that follow, there are 8 lanes per gene. These lanes were organised as follows: lanes 1-2 adult samples, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 samples. The samples shown here are the same as those used in the RT-PCR experiments described in Chapter 3. As such, the ß-actin controls have not been repeated.

4.3.2.2 RT-PCR of CD31 and v α-SMA

mRNA expression of CD31, by RT-PCR (Fig 4.6), appeared to show no difference between any groups with the exception of one PND1 animal (lane 8) where expression was barely detectable. v α-SMA mRNA expression (fig 4.7) appeared higher in E21 and PND1 kidneys (lanes 5-8) and also in one of the transplant samples (lane 3) compared to adult samples.

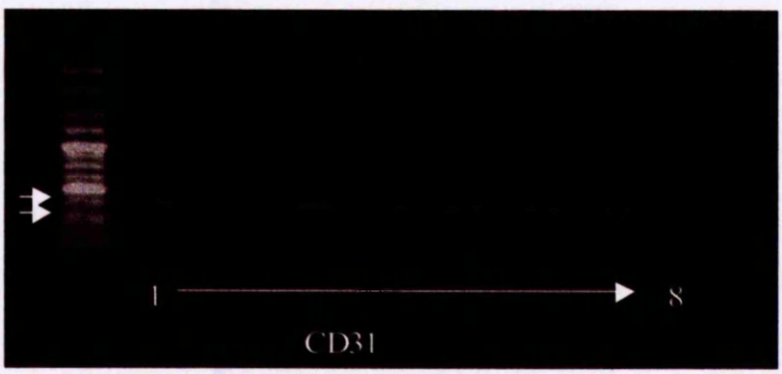

Figure 4.6. mRNA expression of PECAM-1 (CD31) by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of the platelet endothelial cell antigen-1 (CD31). Lanes 1-2 are adult rat kidney, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 kidneys.

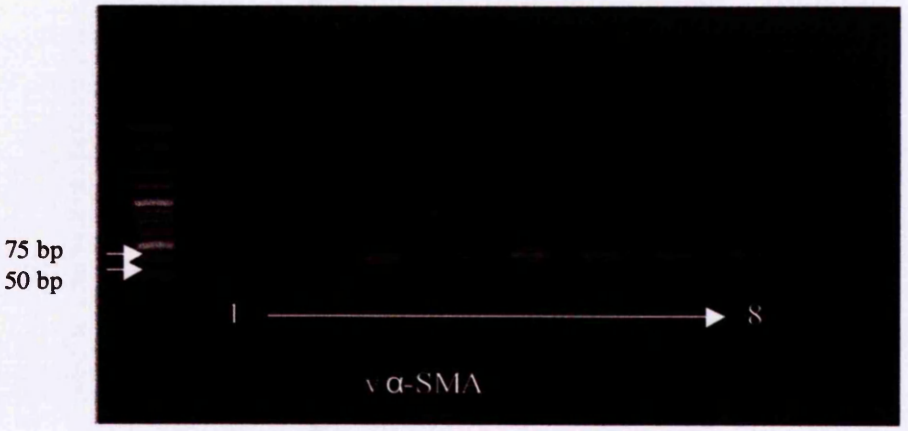

Figure 4.7. mRNA expression of v-αSMA by RT-PCR. A 30-cycle RT-PCR showing mRNA expression of the vascular α-smooth muscle actin (CD31). Lanes 1-2 are adult rat kidney, lanes 3-4 transplanted metanephroi, lanes 5-6 E21 kidneys and lanes 7-8 PND1 kidneys.

4.3.3 Promotion of arteriogenesis

The following section describes the weight of metanephroi and mRNA expression, by qPCR, of CD31, v α-SMA, UT-A1,2 and 3, AQP1 and 2, ENaC, NKCC2 and AT_2R. The two groups are as follows; transplants that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control, n = 6), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL MCP-1 and 1 ng/mL GM-CSF prior to transplant (Tx experimental, n = 6). Adult control kidneys (n = 3) were used to standardise the qPCR results as in chapter 3.

4.3.3.1 *Metanephroi weights*

There was no statistical difference between the weights of the metanephroi (Table 4.1) in the Tx control and Tx experimental groups ($P = 0.088$).

Group	Metanephroi weight (mg)
Tx control ($n = 6$)	40.0 ± 3.4
Tx experimental ($n = 6$)	31.7 ± 2.8

Table 4.4. Weights of metanephroi following explantation. Metanephroi were explanted 3 weeks post-transplantation. Groups are as follows; transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx experimental). All values are expressed as mean ± S.E.M. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

4.3.3.2 *mRNA expression of CD31 and v α-SMA*

Expression of CD31 mRNA (Fig 4.8), in both the Tx control and Tx experimental group, was around twice that of adult Lewis rat kidneys. Values for the control and experimental groups were comparable and showed no significant difference ($P = 0.135$). v α-SMA mRNA expression (Fig 4.9) in both the 2 transplant groups was comparable to adult samples. However, there was no statistical difference in expression between the Tx control and Tx experimental groups ($P = 0.142$)

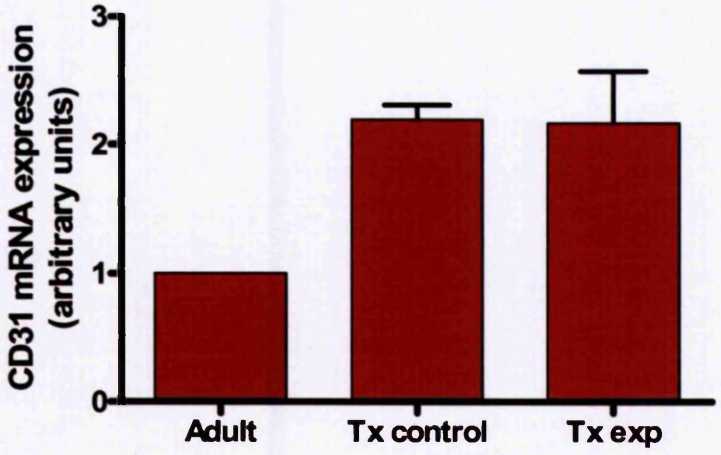

Figure 4.8. mRNA expression of CD31 in control and experimental transplanted metanephroi by qPCR. mRNA expression of platelet endothelial cell adhesion molecule-1 (CD31) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx experimental). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test following $\log_{10}$ transformation of data. $P < 0.05$ was deemed significant.

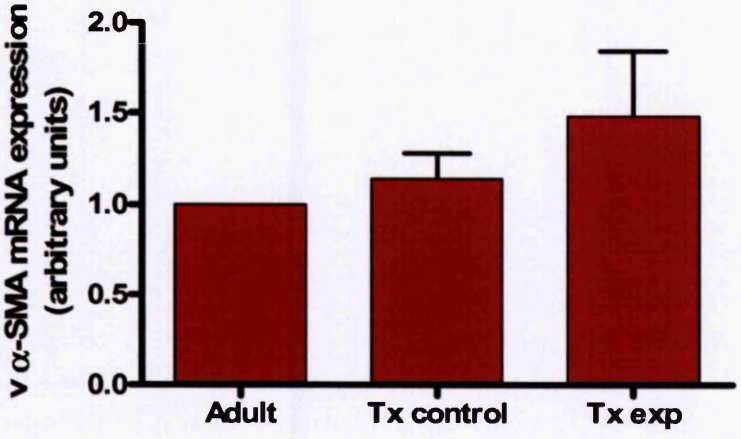

Figure 4.9. mRNA expression of v-αSMA in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of vascular α-smooth muscle actin (v α-SMA) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx experimental). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

4.3.3.3 mRNA expression of urea transporters A1,2 and 3 (UT-A1,2 and 3)

UT-A1/2 mRNA expression (Fig 4.10) was very low in both the Tx control and Tx experimental groups compared with adult samples, with expression around 2-4 % of the levels found within adult kidney. Despite this low expression, there was a statistical difference between Tx control and Tx experimental transplants, with the Tx experimental group having a higher expression of UT-A1/2 ($P = 0.035$).

UT-A1/3 mRNA expression (Fig 4.11) was not detectable within either Tx control or experimental groups, 3 weeks post-transplant.

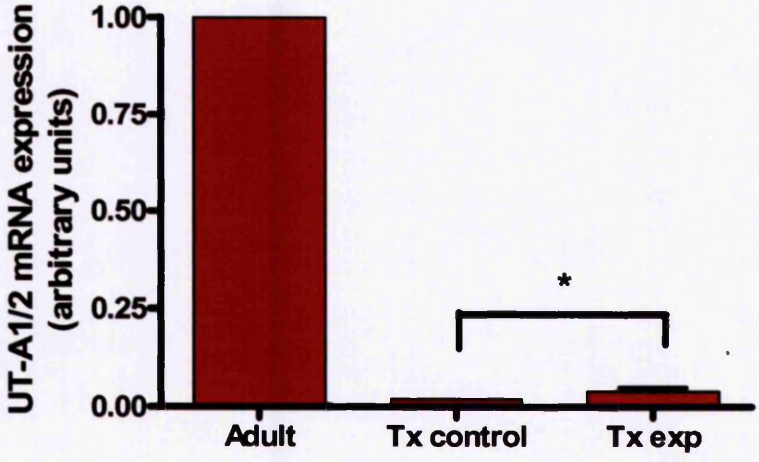

Figure 4.10. mRNA expression of UT-A1 and 2 in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of urea transporter A1 and 2 (UT-A1/2) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant. * $P < 0.05$.

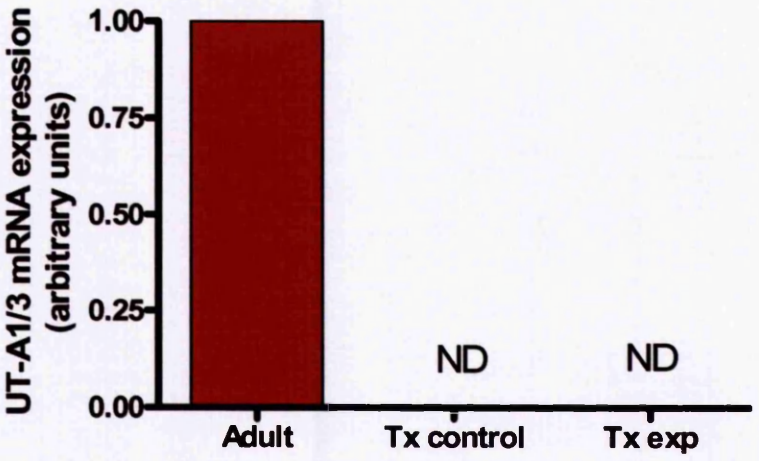

Figure 4.11. mRNA expression of UT-A1 and 3 in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of urea transporter A1 and 3 (UT-A1/3) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. ND; not detected.

4.3.3.4 mRNA expression of aquaporin 1 and 2 (AQP1 and 2)

AQP1 mRNA expression (Fig 4.12) in the Tx control group was around 24% of adult levels, whilst transplants in the experimental group had expression levels around 46% of adult samples. Despite this apparent difference between the two transplant groups, it failed to reach statistical significance (P = 0.064). Relative AQP2 mRNA expression (Fig 4.13) appeared much lower than AQP1 with values around 3% of adult levels for both groups. There was no significant difference between the Tx control and Tx experimental groups (P = 0.840).

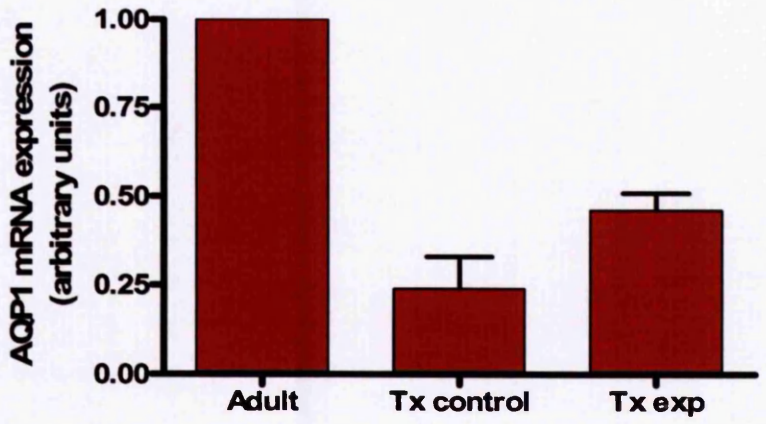

Figure 4.12. mRNA expression of AQP1 in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of aquaporin 1 (AQP1) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

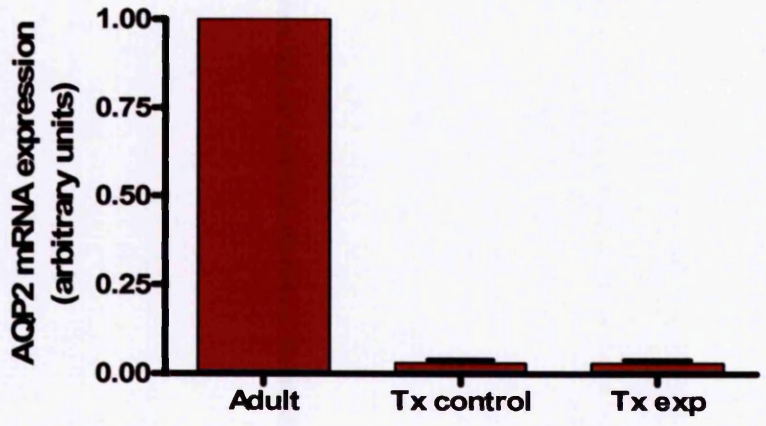

Figure 4.13. mRNA expression of AQP2 in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of aquaporin 2 (AQP2) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

4.3.3.5 *mRNA expression of epithelial Na channel (ENaC) and Na-K-2Cl cotransporter type 2 (NKCC2)*

ENaC mRNA expression (Fig 4.14) was not significantly different between Tx control and Tx experimental groups (P = 0.137) with expression only around 3-4% of adult expression levels. This pattern was also observed for NKCC2 (Fig 4.15) with mRNA expression only around 4% of adult levels in both transplant groups. There was no significant difference in NKCC2 expression between Tx control and Tx experimental groups (P = 0.710).

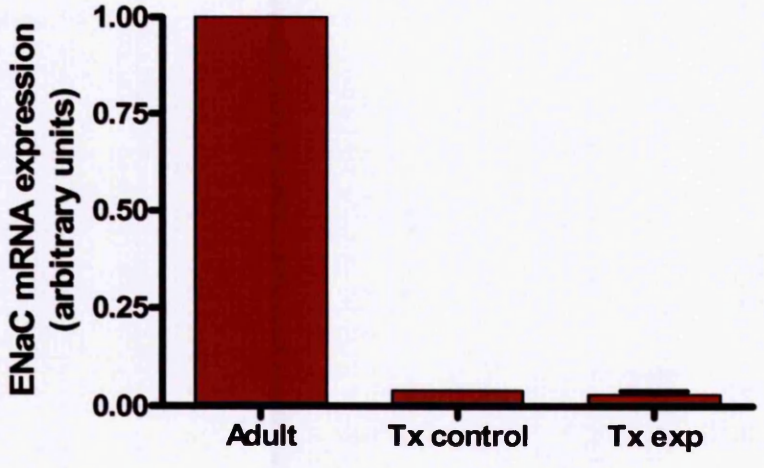

Figure 4.14. mRNA expression of ENaC in control and experimental transplanted metanephroi by qPCR mRNA expression, by Taqman quantitative RT-PCR, of epithelial Na channel (ENaC) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

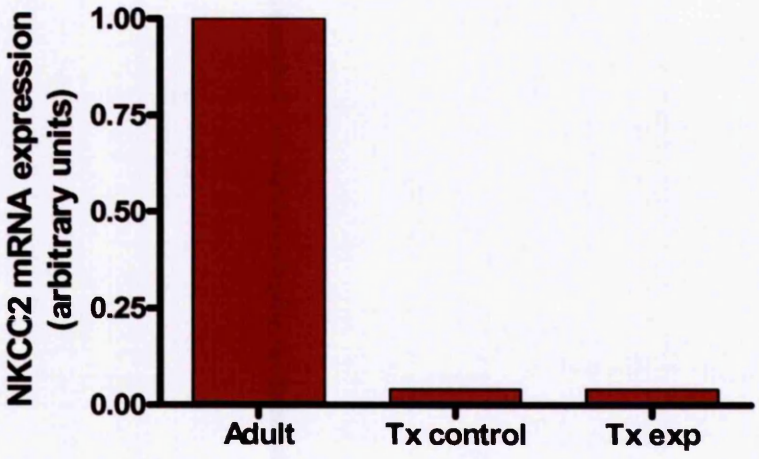

Figure 4.15. mRNA expression of NKCC2 in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of Na-K-2Cl cotransporter type 2 (NKCC2) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

4.3.3.6 mRNA expression of angiotensin II type 2 receptor (AT_2R)

mRNA expression of AT_2R (Fig 4.16) was around 60-80 times higher in both transplant groups compared to adult rat kidneys. However, there was no significant difference in the expression of AT_2R between Tx control and Tx experimental groups ($P = 0.163$).

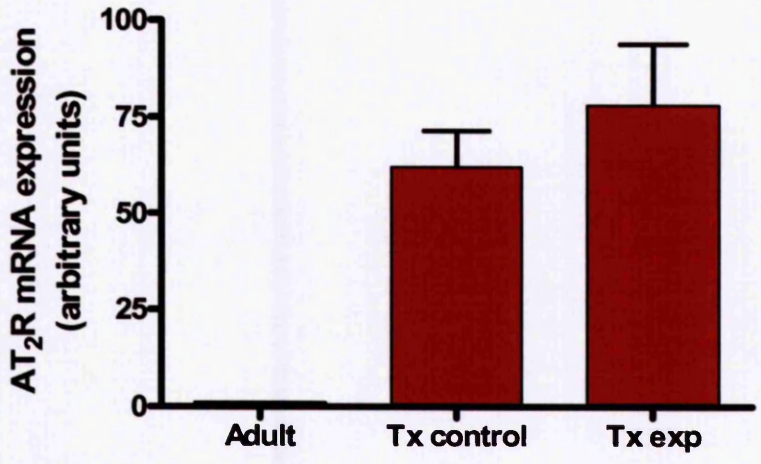

Figure 4.16. mRNA expression of AT_2R in control and experimental transplanted metanephroi by qPCR. mRNA expression, by Taqman quantitative RT-PCR, of angiotensin II type 2 receptor (AT_2R) in transplanted metanephroi that have been incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that have been immersed in 12–growth factor cocktail plus 1 µg/mL monocyte chemoattractant protein-1 (MCP-1) and 1 ng/mL granular monocyte-colony stimulating factor (GM-CSF) prior to transplant (Tx exp). All values are expressed as mean ± S.E.M and are compared with adult expression levels, assigned an arbitrary value of 1. Statistical analysis was by independent samples t test. $P < 0.05$ was deemed significant.

4.4 Discussion

4.4.1 Comparison of RECA-1, CD31 and v α-SMA within transplanted metanephroi, E21 and PND1 animals

Studies have shown, by immunohistochemistry, that RECA-1 protein is first observed in the embryonic rat kidney at E14, with increased expression towards birth and high expression present within the adult kidney (Ricono et al., 2003). Similarly, CD31 is also expressed from E14 in the rat and is highly expressed within adult rat kidney, particularly within the glomeruli (Ricono et al., 2003). Vascular α-smooth muscle actin was first detectable in rat kidney vasculature at E17 and is highly expressed after birth and maintained within the adult kidney (Carey et al., 1992).

All of these studies suggest that protein expression of RECA-1, CD31 and v α-SMA should be visible in transplanted metanephroi, E21 and PND1 kidneys. This was indeed the case. Likewise, there have been recent reports of RECA-1 expression within transplanted metanephroi, with immunohistochemical staining visible within transplants between 5-8 weeks post-transplantation (Takeda et al., 2006). Interestingly, these metanephroi were from pigs rather than rats. Hence this report shows that rat endothelial staining was present within the porcine transplants, suggesting that the source of the endothelium was from the host, via angiogenesis, rather than from the donor organ, via vasculogenesis. This was confirmed by the lack of porcine-specific CD31 staining within the transplants. The transplants in the current study stained for both anti-rat

CD31 and RECA-1, highlighting the presence of endothelial cells within transplanted metanephroi. Clearly, in this case, it is not possible to identify the origin of the endothelial cells detected in the metanephroi by the markers. Nonetheless, the data suggest that vascularisation of the transplant has occurred post-transplantation. CD31 mRNA expression in the transplants was comparable with adult expression levels, although caution is required when interpreting this result given the large variation observed. This large variation exemplifies the varying maturity levels of the transplants. As a result of this large variation, the apparent difference in CD31 mRNA expression between transplanted metanephroi and E21 and PND1 animals was not statistically significant. This suggests that CD31 mRNA expression in transplanted metanephroi may actually be closer to developing, rather than adult, expression levels.

mRNA expression of v α-SMA was comparable in all groups suggesting that, per µg of RNA, there were similar levels of vascular smooth muscle expression in transplants, E21 and PND1 kidneys by comparison with adult levels. It has already been noted that during development of the kidney, v α-SMA is highly expressed at levels similar to those seen in adult (Carey et al., 1992): this is confirmed by the current findings. The fact that transplanted metanephroi had v α-SMA mRNA expression comparable with other developmental groups suggests that the vasculature in the kidney was only as well developed as E21 and PND1 kidneys up to 4 months post-transplant.

4.4.2 Arteriogenesis study

In the following section, it must be reiterated that the transplanted metanephroi were explanted 3 weeks post-transplant. Thus, comparisons with mRNA expression in transplanted metanephroi in Chapter 3 and earlier in Chapter 4 (explanted around 4 months post-transplant) also take into account this difference in 'age' of the transplant. The two groups studied are as follows: transplants that were incubated in the 12-growth factor cocktail prior to transplant (Tx control), and transplants that were incubated in 12–growth factor cocktail plus 1 μg/mL MCP-1 and 1 ng/mL GM-CSF prior to transplant (Tx experimental).

4.4.2.1 CD31 and v α-SMA

mRNA expression of CD31 in Tx control and Tx experimental groups was comparable, at around double the expression of adult control kidneys. The most likely reason for this elevated expression is that angiogenesis was still occurring in the transplanted metanephroi and so endothelial markers such as CD31 were highly expressed. Further evidence for this can be seen when looking at CD31 expression in transplants explanted 4 months post-transplant, which was comparable with adult control levels. This suggests that between 3 weeks and 4 months post-transplant, vascularisation of the transplant had slowed/ceased and CD31 expression was maintained at adult levels; hence relative mRNA expression drops from a value of 2 to around 1.

v α-SMA showed a similar trend to CD31, with no difference in mRNA expression between Tx control and Tx experimental groups. Expression of v α-SMA mRNA was comparable with adult levels, suggesting that between 3 weeks and 4 months post-transplant, further recruitment of smooth muscle cells was not occurring. Hence, although angiogenesis may still have been occurring around 3 weeks post-transplant, it appears that arteriogenesis had ceased. This suggests that, despite the transplants possessing the ability to form smaller arteriolar-type vessels, they appear to lack the ability to recruit the necessary quantity of smooth muscle cells to form larger conductance arteries, leading to reduced blood flow within the kidney. However, it is worth reiterating that expression levels of CD31 and v α-SMA were equivalent to the levels observed in kidneys of a similar size (E21 and PND1 kidneys).

In conclusion, it appears that dosing the transplants with MCP-1 and GM-CSF did not improve arteriogenesis. This may have been due, at least in part, to the relatively low doses used or the brief (1 hour) incubation period. The doses, as described, were comparable with those chosen for the other growth factors, in relation to the EC_{50} value. However, it was decided that doses should be at the lower end of the scale given the fact that MCP-1 and GM-CSF both recruit macrophages, which are known to play a role in the rejection of transplants (Land et al., 1971; Hancock et al., 1993; Heemann et al., 1993; Beckmann et al., 2006). Since it has been shown that a 1-hour incubation in the 12-growth factor cocktail enhances the growth of metanephroi (Rogers et al., 1998; Rogers and Hammerman, 2001a), it was felt that this would be sufficient time for incubation with

MCP-1 and GM-CSF. However, it is very difficult to gauge exactly what 'dose' the metanephroi were receiving since the pharmacokinetics were not measured. It may be that a longer treatment with MCP-1 and GM-CSF would yield better data. Although MCP-1 and GM-CSF did not appear to improve arteriogenesis in the transplanted metanephroi, the question remained as to whether they increased the maturity of the tubules. This was assessed by quantifying mRNA expression levels of key channels and transporters, previously examined in Chapter 3.

4.4.2.2 UT-A 1,2 and 3

UT-A1 and 2 mRNA expression was significantly higher in the Tx experimental group compared to controls. This result, however, must be treated with a degree of caution given the extremely low expression levels that were present. Despite this, it appears that dosing the transplants with MCP-1 and GM-CSF may have resulted in increased expression of UT-A1 and 2. This was not the case for UT-A1 and 3 where there was a complete lack of mRNA expression. These results would therefore suggest that it is the expression of UT-A2, not UT-A1, that has increased. This increase in UT-A2 expression occurred despite there being no apparent increase in smooth muscle recruitment and arteriogenesis. As UT-A2 is known to be important for urea recycling within the thin descending limbs by counter exchange (Trinh-Trang-Tan and Bankir, 1998) it is likely that increased UT-A2 expression would lead to an increase in urea recycling, particularly within the inner stripe of the outer medulla. It is known that UT-A2 expression is decreased following administration of a loop diuretic,

furosemide (Leroy et al., 2000). This is accompanied by a decrease in the osmolality of urine. Thus, an increase in UT-A2 may improve urea recycling and thus increase the osmolality of urine. Further study is required to confirm this hypothesis by measuring the osmolality of urine produced by transplanted metanephroi.

One further observation in the current study worthy of mention was that the mRNA expression of UT-A 1,2 and 3 closely mirrored the results from transplants described in Chapter 3, at around 4 months post-transplant. The lack of UT-A1 and 3 expression at both 3 weeks and 4 months post-transplant strongly suggests that the tubules had ceased to mature, particularly the inner medullary collecting ducts. Likewise, UT-A1 and 2 mRNA expression only appeared to increase slightly between 3 weeks and 4 months post-transplant, suggesting that tubular maturity had almost peaked by around 3 weeks after initial transplantation, albeit still well below the maturity of adult kidney tubules.

4.4.2.3 AQP 1 and 2

AQP1 expression, at first glance, appeared higher in the Tx experimental group compared to controls, however this just failed to reach statistical significance. Despite this, there was a marked difference between AQP1 expression in the Tx experimental group, 3 weeks post-transplant (46% of adult levels), and AQP1 expression in a non-treated transplant, 4 months post-transplant (10 % of adult levels). This suggests that MCP-1 and GM-CSF, through the recruitment of monocytes, had a positive effect on AQP1 expression. The reason for this increase in AQP1 is unknown as this is the

first study of its kind. There are no data in the literature linking monocyte/macrophage recruitment, an inflammatory environment and increased AQP1 expression. It is possible that, since AQP1 is expressed in the thin descending limb, as well as UT-A2, which also showed higher expression in the Tx experimental group, that MCP-1 and GM-CSF were somehow acting locally within the outer medulla of the kidney. This local action may either result in an increase in the number or the maturity of the thin descending limbs and thus AQP1 and UT-A2 show increased expression levels.

It must also be remembered that despite the apparent, observed differences, variation in gene expression levels within the transplants is quite high indicating their different levels of maturity. For instance, there was a difference in AQP1 expression between the Tx controls at 3 weeks post-transplant and the transplanted metanephroi previously examined 4 months post-transplant, which had both been immersed in the 12 growth-factor cocktail only. As such, any interpretation of the data should be treated with a degree of caution. Clearly, in this case, the reasons for increased AQP1 and UT-A2 expression require further study, as will be discussed later.

Dosing the transplants with MCP-1 and GM-CSF appeared to have no effect on AQP2 mRNA expression between Tx control and experimental groups. AQP2 levels in the current study were also slightly lower than those in Chapter 3, (around 10% of adult controls) four months post-transplant. This is the first evidence that the tubules are still maturing beyond 3 weeks post-transplant. This seems logical since AQP2 is known

to reach maximal expression in the rat between 4 weeks to 2 months after birth (Yasui et al., 1996; Yamamoto et al., 1997). Since a transplanted metanephros, 3 weeks post-transplant, would only be equivalent (in time, if not size) to a PND14 rat kidney, AQP2 expression might be expected to continue to increase beyond this time-point.

4.4.2.4 ENaC and NKCC2

ENaC showed a similar pattern to AQP2, with no statistical difference between Tx control and Tx experimental groups, but mRNA expression levels were slightly lower (3-4 % of adult levels) than those observed in the transplants 4 months post-transplant (8% of adult levels). Although these differences are slight, it provides further evidence that the tubules continue to mature beyond 3 weeks post-transplant. Coincidentally, both ENaC and AQP2 were expressed in the collecting duct, albeit in different regions, suggesting that it is the collecting duct that continues to mature in the transplants after 3 weeks post-transplant.

Like ENaC, dosing the transplanted metanephroi with MCP-1 and GM-CSF appeared to have little effect on NKCC2 mRNA expression. There was also no apparent difference between NKCC2 expression 3 weeks post-transplant (4% of adult mRNA expression) and 4 months post-transplant (also 4%). This suggests that NKCC2 mRNA expression in the transplants had reached maximal levels, albeit well below those of adult kidneys.

4.4.2.5 AT_2R

AT_2R mRNA expression was not significantly different between Tx experimental and control groups, suggesting that dosing the transplants with GM-CSF and MCP-1 failed to improve or prolong nephrogenesis within transplanted metanephroi. Although expression of AT_2R mRNA was slightly higher in both groups, compared with transplants 4 months post-transplant, levels were still far closer to adult expression levels than E21 and PND1 kidneys. This indicates that, even 3 weeks post-transplant, nephrogenesis within transplanted metanephroi had ceased. The difference between expression of AT_2R in the current study and the transplants 4 months post-transplant was nominal and nephrogenesis was likely to have stopped in all groups.

4.4.2.6 *Summary of arteriogenesis study*

It would appear from the data presented that treating the transplants with MCP-1 and GM-CSF at the doses used did not promote arteriogenesis. This was apparent by the lack of an increase in expression of either endothelial cells or vascular smooth muscle cells between treated and non-treated transplanted metanephroi. Despite this lack of improvement in arteriogenesis, it appeared that dosing with MCP-1 and GM-CSF did increase expression of AQP1 (compared to the transplants discussed in Chapter 3) and UT-A2. A common feature of these 2 proteins is that they are both expressed in the thin descending limb (tDL) of Henle's loop. Two

hypotheses could explain these results: either MCP-1, GM-CSF, or their combined action, resulted in the development of more or longer tDLs, or the maturity of the limbs was increased. Since there was no difference in the size of the transplants between the Tx control and experimental group, it is unlikely that there were more tDLs in the Tx experimental group (however it is acknowledged that this was not confirmed by a nephron count). Thus, it is more likely that MCP-1 and/or GM-CSF acted in some way to increase the maturity of the tDL. The mechanisms underlying this are unknown and require further investigation, but one interesting observation is that CD44, which is thought to be involved in macrophage activity, has been localised to the kidney, including the tDL (Decleves et al., 2006). Since MCP-1 and GM-CSF are known to be involved in the recruitment of macrophages (Ito et al., 1997; Buschmann et al., 2001) it is logical to assume that macrophages will be present within the transplants. A simple immunohistochemical study would help to examine whether CD44 and macrophages are localised to the thin descending limbs of transplanted metanephroi in the current study, and whether this expression is higher within the Tx experimental group. A positive result here may suggest a role for the macrophages in the increased tubular maturity of the tDL, although the mechanism by which this occurs would require substantial investigation.

In conclusion, the attempt to induce arteriogenesis in transplanted metanephroi by exposure to MCP-1 and GM-CSF was unsuccessful. Arteriogenesis requires the recruitment of smooth muscle cells, so the choice of MCP-1 and GM-CSF was logical. Whether the outcome would

have been different if the dose or time of incubation were increased remains open to speculation. Nonetheless, there is tentative evidence to suggest that MCP-1/GM-CSF treatment enhanced maturation of the tubules, specifically the tDL. If this can be improved in the future, it is possible that the tubules will be able to concentrate urine to a greater degree.

Chapter 5: General discussion

5.1 Fulfilling the aims of the project

Many of the aims of this project have been achieved. Firstly, the *in vivo* clearance studies described the baseline physiology of metanephric transplants in terms of glomerular filtration rate, urine flow rate and, for the first time, renal blood flow and renal vascular resistance. Secondly, the physiology of the transplanted metanephroi was described in terms of their ability to concentrate the urine. This was achieved by simple assays for the measurement of urea and inulin concentrations within urine and plasma, together with a comprehensive profile of the expression of some of the key transporters and channels responsible for the urinary concentration mechanism. Thirdly, glomerular counts and the expression of the angiotensin II type 2 receptor (AT_2R) provided evidence of the premature curtailment of nephrogenesis within the transplants and further evidence of their retarded growth. Finally, an attempt to improve the growth and maturity of the transplants by improving arteriogenesis was assessed. Although arteriogenesis and the predicted subsequent increase in growth of the transplants failed to occur, there was evidence of increased tubular maturity, localised to the thin descending limb. Taken together, these components provide the first comprehensive profile of basal function of transplanted metanephroi.

5.2 Haemodynamics of transplanted metanephroi

Prior to this project, very little was known about the haemodynamics of transplanted metanephroi except that there was a lack of larger conductance arteries both supplying, and within, the transplants. The data presented in Chapter 2 supports this notion, providing evidence of low blood flow and high vascular resistance. It seems likely that improving metanephric blood flow will prove to be the single-most important factor in enhancing the physiological performance of the transplants.

Following transplantation, it has been shown that most, if not all, of the vasculature supplying the metanephros is derived from the host, via angiogenesis (Takeda et al., 2006). What is still unknown is at what time-point these vessels 'reach' the transplants. Clearly if the delivery of blood takes a number of days then the growth of the transplants would be severely affected. Whilst there is little doubt that immersing the transplants in the 12-growth factor cocktail prior to implantation aids in their subsequent growth (Rogers et al., 1998; Rogers and Hammerman, 2001a), this is insufficient to promote 'normal' development. This is despite the fact that there are a number of factors within the 12-growth factor cocktail, such as VEGF and fibroblast growth factor (FGF) which have been shown to play a role in angiogenesis (Gospodarowicz, 1976; Shima et al., 1995). One way to improve the method of growth factor delivery might be to deliver the growth factors constantly over a set period of time via an osmotic mini-pump.

If vascularisation of the transplants could be improved, then overall growth may also increase, given that the supply of blood is a limiting factor for maximal organ growth. Increased growth and blood supply would likely be accompanied by an increase in nephron number and tubular maturity and thus the ability of the transplants to concentrate urine would also be likely to improve. Hence, improvement of metanephric blood flow is crucial for increased physiological function.

Unfortunately, the attempt to improve arteriogenesis in the transplants by dosing with MCP-1 and GM-CSF failed. This could have been due to the dose and/or exposure period, so the use of an osmotic mini-pump may help to improve arteriogenesis and growth of the transplants.

5.3 Nephrogenic state

Explanting transplanted metanephroi following the clearance study showed that the growth of the transplants was retarded; but did the transplants possess a nephron complement appropriate to their size? This was answered through non-biased stereological counting of glomeruli, which showed that per mg of tissue, nephron number was 'normal'; furthermore, there was no difference in nephron number in tissue harvested at 3 weeks post-transplantation and 4-months post-transplant. Measuring AT_2R expression confirmed that nephrogenesis had ceased within the transplants. This demonstrated that not only had nephrogenesis stopped, but that it had probably done so by around 3 weeks after transplantation. This is in complete agreement with the nephron counts

and suggests that improving growth in the first few weeks after transplantation is crucial. This is quite logical considering that nephrogenesis in the normal developing rat kidney continues until around 11 days after birth (Kavlock and Gray, 1982), which is close to the 'actual age' of the transplant after the 3 week transplantation period (around PND14). Thus, even though nephrogenesis in the transplants is retarded, it appears to cease at a 'normal' time point in terms of kidney development.

5.4 The concentration of urine

The ratio of urea in urine:plasma suggested that urea was being concentrated in the urine. Only UT-A2 was present in transplanted metanephroi at measurable levels, with both UT-A1 and 3 expression below the limits of detection. As a result, urea transport was unlikely to be facilitated, certainly within the inner medullary collecting duct (IMCD). This could explain why the urea concentration in metanephric urine was only around 50% of that in the urine produced by adult kidneys. Nonetheless, for urea to be concentrated in the metanephric urine, some form of urea transport must have occurred, be it either passively or facilitated, by UT-A2. Passive diffusion of urea into the tubule, via UT-A2, is likely to occur in the thin descending limb. The removal of water, via AQP2, in the IMCD further concentrates the urea solution in the tubules. However, the lack of UT-A1 and 3, and reduced expression of AQP2, ensures that the urea concentration was reduced compared to adult levels. Furthermore, the fact

that the urine:plasma ratio of urea ($U:P_{urea}$) was much higher than $U:P_{inulin}$ may provide further evidence that the 'concentration' of urea was not solely due to the reabsorption of water, but also through the movement of urea. Despite the fact that urea concentration is limited in the transplants, it is worth reiterating that maximal urine/urea concentrating ability does not occur until several weeks after birth in the rat. Thus, relative to size, it would appear that the metanephroi are 'normal' for their effective age.

The urine:plasma inulin ratios were much lower in transplanted metanephroi than in adult kidneys. Despite this, the inulin concentration was higher in metanephric urine than in plasma, suggesting that some tubular reabsorption of water had occurred. This tubular reabsorption of water would act to increase the concentration of inulin in the urine and also to increase the osmolality of urine. As stated previously, AQP1 and 2, NKCC2 and ENaC are all likely to play a key role in this reabsorption of water. Aquaporin 2 is the main channel by which water is reabsorbed, under the regulation of vasopressin, within the collecting duct in the final concentration process. Thus, the urea concentrations, inulin ratios and presence of UT-A2, AQP1 and 2, NKCC2 and ENaC all suggest that water and salt were being reabsorbed and that the urine produced by transplanted metanephroi is more concentrated than blood/plasma. All of these examples indicate that, though still very immature, the tubules of the transplants are performing some of their normal function.

5.5 General summary

It is important to reiterate that all of the clearance data were derived from animals with a single transplanted metanephros connected to the hosts urinary system. The GFR and RBF measurements were obviously much lower than adult kidneys, but it is possible that multiple transplants could add function in a cumulative manner. It has already been shown that anephric animals surviving on 2 transplanted metanephroi lived significantly longer than animals surviving on a single, transplanted metanephros (Marshall et al., 2006 *In press*). Haemodynamic measurements were not made in this study but it is reasonable to speculate that the total GFR and RBF of 2 transplants exceeded that of the single transplants reported in this thesis. Cumulative function may be much easier to achieve in larger animal models where the connective surgery is simpler due to the larger scale involved.

It is probably not necessary to achieve the level of function of an adult kidney in transplanted metanephroi, in order for this method of renal replacement therapy to be viable in a clinical setting. For instance, taking into account that dialysis is only performed around 3 times per week, for a period of several hours, this equates to an average GFR of around 10-15 % of that of a normal kidney. Animal studies show that normal renal function can be achieved with as few as 30% of functioning nephrons (Guyton and Hall, 2000). Since it has been shown that transplanted metanephroi have GFRs of up to 5-10% of adult levels (Rogers et al.,

1998; Marshall et al., 2005), it appears that this technology may indeed be viable in the future.

Another important point to make is that, even though their growth is retarded, the transplants are functioning appropriately for their size. That is to say that both clearance parameters measured *in vivo* and the expression of key transporters and channels are comparable with published data on kidneys in the immediate postnatal period. This fact alone is very encouraging as it suggests that improving blood supply and growth of the transplant will likely lead to a comparable increase in nephron number, tubular maturity and overall physiological function.

To summarise, the data obtained thus far are very positive. Not only has it been confirmed that E15 rat metanephroi are able to grow and show physiological function following transplantation into a rat host, but it has also been shown that this function is comparable with a kidney of similar size. Similarly, it has been shown that the tubules of the transplants, although still immature, do express some of the transporters and channels required for the urine concentration process and that these transporters are functional and capable of concentrating urea and inulin in the urine. The major message from this study is that whilst transplanted metanephroi do show measurable physiological function, this function is not currently sufficient to sustain life.

Despite this fact, there is encouraging evidence which showed that anephric rats survived on transplanted metanephroi alone for up to 60 hours longer than anephric controls (Rogers, 2004). To date, survival time has been extended to a maximum of 7-8 days. It must also be

remembered that dialysis has been shown to filter blood at an equivalent efficiency of 10-15% of that of a normal, functioning adult kidney (Kuhlmann et al., 2001). Data published to date show that the transplanted metanephros, per gram of kidney weight, has a GFR equivalent to 11% of the GFR of an adult kidney (Marshall et al., 2005). Thus, if the mass of renal tissue can be increased, then the filtration capacity may be improved to a point where life can be sustained. With these points in mind it becomes clear that transplanting metanephroi may well have excellent potential as a future form of RRT in man.

5.6 Future work

As has already been suggested in this section, much of the future work on this project is likely to focus on the theme of improving blood flow to the transplants. This may take the form of another trial involving dosing the transplants with MCP-1 and GM-CSF at higher doses, or using an osmotic mini-pump to deliver a constant infusion over a period of several hours or days. The realisation that the transplants are performing 'normally' comparable to size suggests there may be little scope to improve their physiological function without increasing mass. However, there are some avenues worthy of further investigation that could result in an improvement in tubular handling of ions and water, if not GFR and RBF.

One such option is the administration of a loop diuretic, such as furosemide, bumetanide or piretanide. These drugs have all been shown to exert a diuretic effect by inhibiting NKCC2 (Hannaert et al., 2002) and

hence the reabsorption of salt and water in the thick ascending limb. Administration of such drugs may encourage a diuresis, albeit a limited one considering the low expression of NKCC2 in the transplants, and thus improve the low urine flow rates (UVs) observed. Similarly, angiotensin II is also known to play a role in salt and water reabsorption in the kidney, particularly within the proximal tubule and thick ascending limb (Lerolle et al., 2004). In the thick ascending limb, angiotensin II is known to cause increased expression of the Na^+/H^+ exchanger 3 (NHE3) and NKCC2 (Kwon et al., 2003) leading to increased absorption of NaCl. Angiotensin II, as a potent vasoconstrictor can also act to increase GFR, largely by the constriction of the efferent arterioles, via AT_1R (Frega et al., 1980; Kimura et al., 1997). Blocking the angiotensin receptor AT_1R, with such drugs as valsartan (Wood et al., 1993), would also lead to a diuretic effect and an abolishment of the vasoconstrictive effect on the efferent arterioles. Conversely, dosing the transplants with angiotensin II may aid maturation of the thick ascending limb, since expression of NHE3 and NKCC2 is partly regulated by angiotensin II. Whilst this would likely result in a lower volume of urine, the urine produced may be more concentrated as a result of the increased osmotic gradient produced in the medulla via the actions of NKCC2 and NHE3.

There are also numerous other considerations regarding transplanted metanephroi that have to be addressed. These include whether transplants excrete electrolytes at a 'normal' rate comparable to their size and whether they are able to handle glucose appropriately. Also to be considered is whether the transplants show further signs of normal kidney

physiology such as the secretion of biologically active vitamin D and erythropoietin and whether the transplants perform their expected role in regulating acid/base balance. A further examination of transplanted metanephroi should assess whether they are able to respond appropriately to physiological challenges such as salt/water overloading or shortage (dehydration). Finally, it would be interesting to see whether transplanted metanephroi responded to drugs such as angiotensin converting enzyme (ACE) inhibitors and loop diuretics, since these are commonly used drugs in the effective control of blood pressure.

All of the experiments described in this thesis were performed on syngeneic rat-rat transplants. However, if this technology is going to be taken forward to a clinical setting, decisions have to be made on the source of donor tissue. Initially it was suggested that pig metanephroi may be transplanted into humans. However, it has been shown previously that transplantation of metanephroi across the species barrier requires either substantial levels of immunosuppression or transplantation into immunodeficient hosts (Dekel et al., 2003; Takeda et al., 2006). Although it is likely that human-human transplants would also require substantial levels of immunosupression, long term acute vascular rejection would be less likely than that arising through the transplantation of porcine metanephroi. In a human model, due to the larger scale involved, there may also be the potential to connect vasculature from the host directly to the metanephric transplant, in order to improve the chance of growth.

If human metanephroi are used in the future, the problem of supply and demand arises once more. Of the 185,000 pregnancies terminated per

year in the UK, 88% of these occurred in the first 3 months of pregnancy (Department of Health), so it appears that there are potentially human metanephroi 'available' for transplantation. Preliminary studies have shown that transplanted 7-10 week old human metanephroi undergo nephrogenesis following transplantation into immunocompromised mice (Dekel et al., 2003).

Clearly, such an approach raises enormous ethical issues. Not least, the question of whether scientists and clinicians have the 'right' to approach mothers undergoing an abortion, or whether human embryonic tissue can be used in this manner. Despite these ethical considerations, purely from a scientific perspective, the use of human metanephroi is likely to result in an improved chance of creating a functional, chimeric, life-supporting organ to support/replace the current use of whole organ transplantation, the ultimate aim of the whole project.

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