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Uteroplacental insufficiency in rats induces renal apoptosis and delays nephrogenesis completion

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Short Title – Growth restriction delays nephrogenesis

Abstract

Aim: Uteroplacental insufficiency in rats reduces nephron endowment, leptin concentrations and programs cardiorenal disease in offspring. Cross-fostering growth restricted (Restricted) offspring onto a mother with normal lactation restores leptin concentrations and nephron endowment. This study aimed to determine if the reduced nephron endowment in Restricted offspring is due to delayed glomerular formation and dysregulation of renal genes regulating branching morphogenesis, apoptosis or leptin signalling. Furthermore, we aimed to investigate if cross-fostering Restricted offspring onto Control mothers could improve glomerular maturation and restore renal gene abundance.

Methods: Uteroplacental insufficiency was induced by bilateral uterine vessel ligation (Restricted) or sham (Control) surgery on gestation day 18 (E18). Kidneys were collected at E20, postnatal day 1 (PN1) and PN7. An additional cohort was cross-fostered onto separate mothers at birth and kidneys collected at PN7.

Results: Kidneys were lighter in the Restricted group, but weight was restored with cross-fostering. At E20, *Bax*, *Flt1* and *Vegfa* abundance were increased in Restricted offspring, while *Ret* and *Bcl2* transcripts were increased only in Restricted females. At PN7, *Gdnf* and *Ret* abundance were higher in Restricted offspring, as was *Casp3*. Restricted offspring had a wider nephrogenic zone with more immature glomeruli suggesting a delayed or extended nephrogenic period. Cross-fostering had subtle effects on gene abundance and glomerular maturity.

Conclusion: Uteroplacental insufficiency induced apoptosis in the developing kidney and delayed and extended nephrogenesis. Cross-fostering Restricted offspring onto Control mothers had beneficial effects on kidney growth and renal maturity, which may contribute to the restoration of nephron endowment.

Keywords - branching morphogenesis, growth restriction, kidney, leptin, vasculogenesis.

Introduction

Epidemiological studies and animal models suggest that fetal adaptations following a compromised intrauterine environment during critical stages of development occurs at the expense of organ development, resulting in a low birth weight and long-term disease risk.[1-3] The fetal kidney is particularly susceptible to maternal perturbations,[4] with a low birth weight being associated with a reduction in nephron endowment.[5-9] As renal function plays a central role in maintaining fluid homeostasis in the adult, impaired kidney development is thought to play a major role in the developmental programming of cardiorenal disease. Being born with a low nephron endowment increases the risk of developing a multitude of diseases in adulthood,[1,8-15] with sex-specific differences.[16] It is important to recognise that this nephron deficit is permanent as after the completion of nephrogenesis no new nephrons can be formed. Nephrogenesis completes prior to birth in humans and 7-10 days after birth in rodents.[17]

Kidney development, including nephrogenesis, is a complex but highly regulated process. While many factors have been identified as important regulators of normal renal development,[6] less is known about the regulation of these factors during prenatal insults. Genes regulating renal branching morphogenesis, renal growth, cellular proliferation and apoptosis are altered in developing rat kidneys following maternal perturbations.[10,11,18-21] These studies suggest that multiple pathways contribute to the reduction in nephron endowment. We have previously demonstrated that improving the postnatal environment, through cross-fostering, restores nephron endowment and prevents disease,[14,15,22,23] which may be due to the benefits of accelerated early postnatal growth.[14,15,22,24-26] However, no studies to date have investigated whether these improvements in

nephron endowment are due to changes in branching morphogenesis, renal growth, cellular proliferation and/or apoptosis.

Recent studies highlight that the adipokine leptin plays a role in fetal development, with its concentration peaking in mammals during the completion of organogenesis.[27] Leptin antagonism during the leptin peak (from postnatal days (PN) 2-13) reduces glomerular number and size,[28] highlighting that leptin plays a role in nephron endowment. Additionally, maternal leptin is transferred to the pup via the milk and is absorbed into the blood stream where it may have a bioactive function in the pup.[29] We have recently demonstrated that leptin is reduced in Restricted offspring at PN7, and is restored with cross-fostering;[23] highlighting a potential role for leptin in nephron endowment.

In this study, we aimed to elucidate the pathways through which uteroplacental insufficiency results in decreased nephron endowment by examining key factors involved in branching morphogenesis, renal growth, angiogenesis, apoptosis and leptin signalling at E20 and PN1. As nephrogenesis continues postnatally, during the first week in the rat, we also examined the kidneys of offspring at PN7 in order to examine the contribution of the early postnatal period on nephrogenesis and if these changes are prevented with cross-fostering. We hypothesise there will be sex-specific dysregulation of genes associated with kidney development in growth restricted pups that may help explain why females are protected from developing cardiorenal disease. In males, we predict that these gene changes will be prevented by cross-fostering. We will also test the novel hypothesis that leptin plays a role in the impaired nephron endowment in growth restricted pups.

Results

Body and kidney weights

Uteroplacental insufficiency surgery on E18 (term = 22 days) reduced offspring body and kidney weights in males and females at all ages investigated (Table 1). Male body weight was greater than females at E20 (P = 0.050; two-way ANOVA), but not at PN1 or PN7 (Table 1). Kidney-to-body weight ratio in Restricted offspring was decreased at PN1 (-8%) and increased at PN7 (+3%), with no changes at E20 (Table 1). Female offspring had increased kidney-to-body weight ratio at PN1 and PN7 compared to males.

In the cross-fostering groups, male and female *Restricted-on-Control* and *Restricted-on-Restricted* offspring remained smaller than Controls, with a similar pattern observed in female absolute kidney weight (Table 2). *Restricted-on-Control* offspring had increased absolute kidney weight compared to *Restricted-on-Restricted*, but did not catch up to *Control-on-Control* (Table 2). *Restricted-on-Control* and *Restricted-on-Restricted* male offspring had increased kidney-to-body weight ratio compared to Controls, with no differences observed in females (Table 2). There were no differences between sexes.

Branching morphogenesis genes

At E20, *Ret* was upregulated in Restricted females compared to Control females (+541%), with no differences in *Gdnf* and *Gfra1* abundance (Table 3). *Gdnf, Ret* and *Gfra1* abundance were not different between Treatments or Sexes at PN1 (Table S1). However, transcript abundance of *Ret* (+97%; Fig 1a) and its ligand, *Gdnf* (+42%; Fig 1b), were increased in Restricted offspring compared to Control at PN7, with no changes in *Gfra1* (Table S2). This increase in *Gdnf* mRNA abundance was

supported by an increase in GDNF protein expression in Restricted females (+49%; Fig 1c). In addition, males demonstrated higher relative *Gdnf* mRNA abundance than females at PN7 (Fig 1b). *Tgfb1* and *Wnt11* mRNA abundance were not different at E20 (Table 3), PN1 (Table S1) or PN7 (Table S2).

Cross-fostering did not alter *Ret* (Fig 1d) or *Gdnf* (Fig 1e) mRNA abundance. Interestingly, *Tgfb1* was increased in *Control-on-Restricted* male offspring compared to *Control-on-Control* (+125%), with no changes in *Wnt11* (Table S3).

Angiogenic genes

At E20, *Vegfa* (+96%) and *Flt1* (+277%) transcripts were upregulated in Restricted fetuses compared to Controls, with no differences in *Kdr* (Table 3). At PN1, *Vegfa*, *Flt1* and *Kdr* mRNA abundance was not different between Treatments and Sexes (Table S1). At PN7, *Vegfa* mRNA abundance was increased in Restricted offspring compared to Control (+24%; Fig 2c), with no Treatment or Sex effects in *Flt1* (Fig 2a) or *Kdr* (Fig 2b). However, no changes were observed in VEGFA protein expression in Restricted females at PN7 (Fig 2d).

Flt1 was increased in *Control-on-Restricted* female offspring compared to *Restricted-on-Control* (+260%), with no changes in males (Fig 2e). Whereas, *Kdr* tended to be increased in *Control-on-Restricted* male offspring compared to *Control-on-Control* although this did not reach statistical significance (+145%), with no changes in females (Fig 2f). Cross-fostering did not alter *Vegfa* mRNA abundance (Fig 2g).

Proliferation and apoptosis genes

At E20, *Bcl2* (females only; +423%) and *Bax* (both sexes; +204%) mRNA abundance was increased in Restricted offspring compared to Control (Table 3). At PN7, whilst there was no difference in *Bcl2* and *Bax* abundance (Table S2), *Trp53* (+33%; Fig 3a) and *Casp3* (+22%; Fig 3b) mRNA abundance was increased in Restricted offspring compared to Controls, with males exhibiting higher abundance of *Trp53* than females (Fig 3a). This increased *Casp3* transcript abundance translated to a qualitative increase in cleaved caspase-3 immunoreactive staining in the kidneys from Restricted (Fig 3d) offspring compared to Control (Fig 3c). While there was evidence of cleaved caspase-3 in most cell types of the cortex, the most intense staining was identified in the tubular cells rather than the nephrogenic zone. In addition, there was a trend for a sex effect in *Bcl2* with abundance being higher in the males at PN7, although this did not reach statistical significance (+22%, Table S2; *P* = 0.051).

Cross-fostering Restricted males onto a Control mother restored *Trp53* abundance to *Control-on-Control* values, however this did not reach statistical significance (Fig 3e; p=0.058). *Trp53* was not different in females, however female abundance was greater than males (Fig 3e; one-way ANOVA). *Casp3* abundance tended to be increased in *Control-on-Restricted* female offspring compared to *Restricted-on-Control*, although this did not reach statistical significance (*P* = 0.052; one-way ANOVA) (+150%; Fig 3f). *Casp3* mRNA abundance was not different across Treatments in males (Fig 3f). *Bax* and *Bcl2* abundance were not different across Treatment groups (Table S3).

Leptin signalling genes

At E20, there was an interaction between Sex and Treatment in *Stat5a* abundance, with a post-hoc tests showing abundance was reduced in Restricted females, however this did not reach statistical significance (*P*=0.052, Tukey's post-hoc test; Table 3). There were no differences in *ObR* or *Megalin*

mRNA abundance or the transcript abundance of their downstream signalling targets (*Jak2, Stat3, Socs3, Pi3k, mTOR, Akt3, Ampka* and *Ampkb*) at E20 (Table 3). Despite no changes in *Stat3* mRNA abundance at PN1 (Table S1), at PN7 *Stat3* mRNA abundance was increased in Restricted compared to Control (+31%; Fig 4a). At PN1, *Megalin* mRNA abundance was reduced in Restricted offspring compared to Control (-23%), primarily driven by a large decrease in females (Table S1); however, no changes were observed in *Megalin* transcript abundance at PN7 (Fig 4b). At PN7, *Pi3k* mRNA abundance was reduced in Restricted females compared to Control females (-33%; *P* = 0.047, Tukey's post-hoc test), and was increased in Control females compared to Control males (*P* = 0.023, Tukey's post-hoc test; Fig 4c), which was not evident at PN1 (Table S1). At PN7, there was an interaction in *Ampkb* abundance, however post-hoc analysis was not able to identify where the significance lies (Table S2). There were no changes in *ObR* abundance and other downstream signalling targets of both receptors at PN1 (Table S1) or PN7 (Table S2).

In male offspring, cross-fostering Restricted offspring onto either a Control or different Restricted mother increased *Jak2* compared to *Control-on-Control* (+45%; Table S3). Additionally, *Restricted-on-Control* and *Control-on-Restricted* male offspring had increased *Ampkb* mRNA abundance compared to *Control-on-Control* (+96%; Table S3). Cross-fostering resulted in a reduction in *Megalin* (-35%) and *Pi3k* (-37%) mRNA abundance in all female groups compared to *Control-on-Control* (Fig 4d and f). Cross-fostering did not alter *Stat3* transcript (Fig 4b), *ObR* mRNA abundance or other downstream signalling targets of both receptors (Table S3). In *Control-on-Control* females *Jak2*, *Megalin*, *Pi3k*, *mTOR* and *Ampka* were increased, in *Restricted-on-Control* females *Jak2* and *Megalin* were reduced, and in *Restricted-on-Restricted* females *Ampka* was increased compared to male counterparts (Table S3; Fig 4d and e).

Morphological assessment of nephrogenesis

There was no obvious nephrogenic zone or immature glomeruli in any region of the kidney in the Control group. However, in the kidneys of the Restricted group there were evident comma and s-shaped bodies in all sections examined (data not shown).

Representative kidney sections from male offspring from each of the cross-fostering groups are shown in Figures 5a-d. Cross-fostering Restricted offspring onto a Control mother (*Restricted-on-Control*) partially improved the nephrogenic zone rating in both sexes and width (males only), as they were not statistically different to *Restricted-on-Restricted* (Fig 5e and f, left panels). However, nephrogenic width in female offspring was not restored to *Control-on-Control* values, but were intermediate between *Control-on-Restricted* and *Restricted-on-Restricted* offspring (Fig 5f, right panel).

Discussion

A nephron deficit occurs in offspring following many prenatal perturbations,[9,15,22] however the molecular mechanisms have not been thoroughly examined. We demonstrate that following uteroplacental insufficiency the fetal kidney becomes hypoxic and, along with an inadequate supply of nutrients, this results in slowed fetal and kidney growth, triggering increased *Vegf* abundance and apoptosis. This increased apoptosis during fetal development is likely to contribute to the reduced nephron endowment in Restricted adults. The most interesting outcome of this study was that there was an extension of nephrogenesis in the Restricted offspring, reflected morphologically as well as by an increased abundance of key branching morphogenesis genes. Whilst extending the period of nephrogenesis has the potential to improve nephron endowment Restricted offspring have a reduced nephron number,[15,22] suggesting this elongation of nephrogenesis reflects slowed or

delayed renal development. Interestingly, cross-fostering Restricted offspring onto a mother with normal lactation (Control) prevented the increase in nephrogenic zone width and immaturity of the glomerulus, despite only subtle effects on the abundance nephron formation genes. Taken together, this data indicates that uteroplacental insufficiency alters the normal process of nephrogenesis via multiple factors during both the prenatal and postnatal period, and highlights the benefits of an improved lactational environment on kidney development.

Uteroplacental insufficiency effects on renal growth and development

In our model of uteroplacental insufficiency nephron endowment was reduced by ~50%,[9,15,22] which is consistent with other models of fetal growth restriction[30,31] Importantly, for the first time, we report that kidney size is reduced in Restricted offspring. This is of great relevance as new nephrons cannot be formed after the cessation of nephrogenesis (~1 week of age in the rat). Therefore, any further increase in kidney size after nephrogenesis cessation typically results from renal hypertrophy rather than *de novo* nephron formation.

Down regulation of genes involved in branching morphogenesis are reported in the fetal kidney following maternal undernutrition (E20; 50% food-restriction from E10),[18] whilst altered *Gdnf* and/or *Ret* have been reported following maternal dexamethasone and alcohol exposure.[11,19] Despite few changes in the abundance of branching morphogenesis genes at E20 and PN1 *Gdnf* and *Ret* were increased in Restricted offspring at PN7, suggest branching morphogenesis is ongoing. This could either represent a delay in the completion of nephrogenesis or a compensatory increase in branching within the kidney in an attempt to restore nephron endowment following the prenatal insult; however this does not lead to increased nephron formation.[15,22] In addition, recent data suggest that changes in the rate of ureteric branching, rather than compensatory lengthening of

nephrogenesis, occurs following a perturbation in the developing kidney.[32] When the pool of *Gdnf* expressing renal progenitor cells was ablated at E12.5 there was no compensatory proliferation of the remaining progenitors, but rather a change in the rate of ureteric bud branching.[33] If the same is true in the rat, the increased abundance of *Gdnf* in Restricted offspring may reflect a remaining set of progenitor cells that have differentiated more slowly, resulting in fewer branching events.

Uteroplacental insufficiency effects on angiogenesis

The major angiogenic factor of importance in renal development is *Vegf*, which can be regulated by hypoxia.[34] At E20, renal *Vegfa* and one of its receptors (*Flt1*) were increased, particularly in females, suggesting that uteroplacental insufficiency has induced a relative degree of renal hypoxia. These results are in contrast to those of Baserga *et al.*[35] who identified that uteroplacental insufficiency decreased renal Vegf expression (mRNA and protein) at birth, and increased Vegf expression (mRNA and protein) in adult females. In both models, the nephron deficit occurred in both sexes but only males developed renal disease and hypertension, which suggests the elevated VEGF in adult females[35] may be protective against the development of disease. The reasons for these differences in *Vegf* abundance between the two models are not known, but may reflect differences in the strain of rats, timing of uteroplacental insufficiency, degree of growth restriction (20-25%[35] vs 10-15% in our model) or age at which gene analyses were performed.

Uteroplacental insufficiency effects on proliferation and apoptosis

The most significant gene changes were characterised in factors regulating apoptosis. *Bax* was increased in the kidneys of Restricted fetuses suggesting increased cell death, which is consistent with other models of maternal pertubations.[21,36,37] At PN7, there was also evidence of increased apoptosis in the kidneys of the Restricted offspring, without changes in proliferation. In particular,

Trp53 was increased, which is of great interest as uteroplacental insufficiency alters methylation of the p53 gene in the fetal kidney close to term,[36] suggesting epigenetic alterations may play a role in the renal apoptosis in this model. Caspase-3 is activated during remodelling of the developing kidney as well as in disease states,[38] such as maternal undernutrition.[33] The increased *Casp3* and cleaved caspase-3 we report further suggests that the kidneys of Restricted offspring are immature and still undergoing significant remodelling. Interestingly, in our model apoptosis was not only localised to the nephrogenic zone as previously published,[39] but was also present through much of the renal cortex and medulla.

Uteroplacental insufficiency effects on leptin signalling

There were limited changes in *ObR*, *Megalin* and their downstream signalling targets. Only Restricted females had reduced *Megalin* abundance at PN1, which mechanistically, independently of its role as a signal transducer,[40-43] may increase albumin and protein excretion in the urine.[44] In the early postnatal period the developing rat kidney is still quite immature and the changes in megalin abundance we report may not impair renal protein handling. However, studies in Megalin^{-/-} mice have reduced apical vesicles in the tubules, suggesting a decreased ability of the kidney to reabsorb ligands from the glomerular filtrate.[45] This highlights the need for additional studies to characterise renal protein handling in growth restricted offspring prior to weaning and in adulthood. We cannot also discount at this time changes in ObR or megalin cell surface availability and cell signalling modulation in the kidney. Additional studies are also required to quantify protein expression in the kidney, especially considering leptin's clear ability to impair nephrogenesis.[28]

Cross-fostering effects on nephrogenesis

Cross-fostering Restricted offspring onto a Control mother (*Restricted-on-Control*) increased kidney weight (+12-16%) compared to *Restricted-on-Restricted*, highlighting the benefits of cross-fostering on kidney growth prior to the completion of nephrogenesis. Interestingly, we report a small number of changes in the abundance of genes regulating nephrogenesis in *Restricted-on-Control* offspring. In male offspring we demonstrated increased *Kdr* and *Tgfb1* abundance in *Control-on-Restricted* compared to *Control-on-Control* and in females increased *Flt1* in *Control-on-Restricted* compared to *Restricted-on-Control* offspring, which indicate increased angiogenesis and branching morphogenesis. This finding may indicate an adaptation to prevent any deleterious changes in kidney development in Control offspring in response to the poor lactation environment associated with a Restricted mother;[23] thus maintaining a normal nephron endowment despite a poor postnatal environment. These data further highlight that nephrogenesis is delayed or extended in Restricted offspring. This highlights the need for additional studies at subsequent postnatal ages to identify when nephrogenesis completes in Restricted offspring. Similarly, cross-fostering did not restore the abundance of leptin signalling genes, which may suggest that the adult nephron deficit in these animals[15,22] is not modulated postnatally by leptin signalling via ObRb and Megalin, activating the JAK/STAT or PI3K pathways, at least at these ages. As JAK and PI3K are upstream of other signalling cascades, including MAPK and Forkhead box 0, future studies should investigate the effect of uteroplacental insufficiency on these signalling pathways.

Conclusion

In conclusion, uteroplacental insufficiency in the rat results in offspring growth restriction during pregnancy and lactation. This was associated with a decrease in kidney weight and increased renal apoptosis, with minimal changes in leptin signalling. Although there was an extension in nephrogenesis in Restricted offspring, this likely represents slowed renal development rather than

compensatory nephrogenesis. This increased apoptosis and slowed/delayed nephrogenesis results in the nephron deficit that is reported later in life and associated with adult cardiorenal disease. Cross-fostering, on the other hand, enhances renal maturity despite few changes in gene abundance. This study highlights an important window of intervention in rodents to enhance offspring growth, organ development and maturation by improving early postnatal nutrition. However, the exact mechanisms that contribute to the improved nephrogenesis in growth restricted rats is still unknown and future studies are required to identify the milk protein/s responsible. It is important to recognise a limitation of rodent studies is that nephrogenesis completes in early postnatal life whereas it completes prior to birth in humans. No studies have characterised if the period of nephrogenesis is extended into the postnatal period in growth restricted humans. If this occurs, then our study highlights the potential benefits of adequate milk nutrition in growth restricted infants.

Materials and Methods

Animals

All experiments were approved by the University of Melbourne Animal Ethics Committee (AEC: 0004138 and 0002081) and the La Trobe animal ethics committee (AEC: 12-42) before commencement following the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes. Wistar Kyoto (WKY) rats (9–13 wk of age) were obtained from the Australian Resources Centre (Murdoch, WA, Australia) and provided with standard food pellets and tap water *ad libitum*. Rats were mated overnight and the presence of sperm in the vaginal smear the following morning indicated successful mating and was taken as day 1 of gestation. On day 18 of gestation (term = 22 days), pregnant rats were randomly allocated into two study groups, Control and Restricted, and underwent bilateral uterine vessel ligation or sham surgery as described previously.[46]

Study 1: Developmental study

On E20, pregnant females (n=12-14 mothers/group) and their fetuses were terminally anesthetized with an IP injection of ketamine and illium-xylazil.[46] The fetuses were removed from the mother, weighed and kidneys dissected. Offspring from the PN1 groups were removed from their mothers (n=12-13 mothers/group) and euthanized by decapitation. Offspring in the PN7 groups remained with their own mothers from birth (n=15-16 mothers/group) before being euthanized by decapitation. Right kidneys were fixed in 10% neutral-buffered formalin, with left kidneys frozen in liquid nitrogen and stored at -80°C. For analyses, kidneys from one male and one female from each litter were chosen with a body weight that was the closest to the litter average, with each sample representing a single animal (i.e. n=1).

Study 2: Cross-fostering

In a separate cohort, Control and Restricted offspring were cross-fostered 1 day after birth onto separate mothers (sham operated (Control) or bilateral uterine vessel ligation (i.e. uteroplacental insufficiency; Restricted) surgery mothers) to yield four treatment groups (*Control-on-Control*; *Control-on-Restricted*; *Restricted-on-Control; Restricted-on-Restricted*; n=8-11 mothers/group) as previously described.[22] Each experimental group had an even offspring sex ratio. On the morning of PN7, offspring were removed from their mother and weighed, then euthanized by decapitation, and kidneys collected.

RNA extraction and nephrogenic genes

Total RNA was extracted from frozen kidneys using commercially available kits by Qiagen (Clifton Hill, VIC, Australia) and Norgen (Cambridge, ON, Canada). RNA was reverse transcribed into cDNA

using kits by Applied Biosystems (Scoresby, VIC, Australia) and Qiagen. Real Time PCR was used to determine relative abundance of genes involved in angiogenesis (*Vegfa*, *Kdr* and *Fltl*), renal growth (*Tgfb1* and *Wnt11*), branching morphogenesis (*Gdnf*, *Gfra1* and *Ret*) and apoptosis and proliferation (*Bax*, *Bcl2*, *Casp3* and *Trp53*). Taqman Gene Expression Assays (Applied Biosystems) or custom-designed Taqman primers and probes were used as described previously.[11,19] Real time PCR was performed as a multiplex reaction with ribosomal endogenous *Rn18s* as a housekeeping gene. The delta delta cycle of threshold (Ct) methodology was utilised to determine relative gene abundance compared to the male Control group. Statistical analysis identified that *Rn18s* was not different between Treatment or Sex.

Leptin signalling genes

Custom RT[2] Profiler PCR Arrays were designed and manufactured by Qiagen for the following leptin transporters and signalling targets of interest using SYBR green as the fluorescent agent as described previously.[23] Real Time PCR was used to determine relative abundance of leptin transporters (*Megalin* and *ObR*) and their downstream signalling targets (*Jak2*, *Stat3*, *Stat5a*, *Socs3* and *Pi3k*, *Akt*, *mTOR*, *Ampka*, *Ampkb*). To compensate for variations in RNA quantity and reverse transcriptase efficiency, mRNA abundance of the genes of interest were normalized to the housekeeping gene Succinate Dehydrogenase subunit A (*Sdha*; NM_130428). The delta delta cycle of threshold (Ct) methodology was utilised to determine relative gene abundance compared to the male Control group.[23] Statistical analysis identified that *Sdha* was not different between Treatment or Sex.

Western blot analysis

Protein was extracted from PN7 kidneys from the developmental study as previously described[47] and Western blotting performed to examine relative protein concentrations of VEGFA (1:1,000; Merck

Millipore, Bayswater, VIC, Australia) and GDNF (1:1,000; Abcam, Melbourne, VIC, Australia). ACTB (1:10,000; Sigma-Aldrich, Castle Hill, NSW, Australia) was used to control for protein loading. 50 µg of total protein was run on 10-12% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked in 3% fish gelatine and incubated at 4°C for 1-4 hours). Densitometry was performed using the Odyssey 2.0 software package (LI-COR Biosciences). Western blots were repeated three times and the mean values presented. We have previously validated the VEGF antibody in the rodent placenta.[47]

Renal morphology

PN7 fixed kidneys from the developmental study underwent standard processing to paraffin and multiple sections were cut at 5 µm (10-12 sections/kidney). Sections were stained with haematoxylin and eosin, and then examined for the presence or absence of a nephrogenic zone. Each slide was graded as having a present nephrogenic zone (as evidenced by comma and s-shaped bodies) or no nephrogenic zone. The observer was blinded to the treatment group. For the cross-fostering study, kidneys were processed as described above. In order to determine if cross-fostering alters glomerular maturity, the blinded observer ranked histological sections based on the definitions described previously[48] that were assigned a score (Table S4) termed nephrogenic zone rating. The width of the nephrogenic zone was also measured as previously described.[48]

Immunohistochemistry

5 µm sections of paraffin embedded fixed PN7 kidneys from the developmental study were dehydrated, subjected to antigen retrieval using a 10 mM sodium citrate solution and endogenous peroxidase activity blocked. On each slide, one section was exposed to a rabbit cleaved caspase-3 antibody (1:300, Cell Signalling Technology; Boston, MA, USA), a second section with an equal

concentration of rabbit IgG and a third section with 3% BSA only overnight. A biotinylated secondary antibody was applied and sections stained using avidin–biotinylated enzyme complex (Vectorlabs; Burlingame, CA, USA) and 3,3'-Diaminobenzidine. Slides were counterstained using haematoxylin.

Statistical analysis

Data are presented as mean ± SEM. Results from the developmental study were compared in each age group using a two-way analysis of variance (ANOVA) with Treatment and Sex as factors. Where an interaction was detected ($P < 0.05$), Tukey's post-hoc testing was performed. Western blot data were analysed with a Student's t-test to determine differences between Control and Restricted. For the cross-fostering study, data were initially analysed using a two-way ANOVA to identify any sex-specific differences. If there was an interaction present, a Student's t-test identified differences in Treatment groups across Sexes. Data were then split by Sex and a one-way ANOVA was performed to determine the main differences across Treatment groups within each Sex, with a Student–Newman–Keuls post-hoc test used to identify significant differences between cross-foster groups.

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Conflict of Interest

The authors declare no conflicts of interest.

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Table 1. Body and kidney weights for Control and Restricted male and female offspring at embryonic day 20 (E20), postnatal day 1 (PN1) and PN7 (n=12-16 litters at each age and treatment). Data were analysed with a two-way ANOVA to determine differences between Treatments and Sexes. Data are expressed as mean ± SEM, where 'ns' is not significant.

	Age	Male		Female		Treatment	Sex	Interaction
		Control	Restricted	Control	Restricted			
Body weight	E20	1.80 ± 0.04	1.51 ± 0.04	1.67 ± 0.05	1.46 ± 0.04	$P = 0.0001$	$P = 0.050$	ns
(g)	PN1	4.29 ± 0.07	3.45 ± 0.11	4.06 ± 0.05	3.47 ± 0.12	$P = 0.0001$	ns	ns
	PN7	10.28 ± 0.20	8.04 ± 0.48	9.70 ± 0.18	7.56 ± 0.30	$P = 0.0001$	ns	ns
Kidney weight	E20	13.0 ± 0.5	10.7 ± 0.7	11.6 ± 0.5	10.3 ± 0.6	$P = 0.004$	ns	ns
(mg)	PN1	41.5 ± 0.8	30.4 ± 0.9	41.0 ± 0.6	32.3 ± 1.5	$P = 0.0001$	ns	ns
	PN7	123.4 ± 2.8	98.6 ± 4.8	124.1 ± 3.1	97.6 ± 2.9	$P = 0.0001$	ns	ns
Kidney/body weight	E20	7.23 ± 0.29	7.07 ± 0.41	6.95 ± 0.27	7.10 ± 0.41	ns	ns	ns
(mg/g)	PN1	9.66 ± 0.11	8.84 ± 0.21	10.10 ± 0.13	9.32 ± 0.30	$P = 0.0002$	$P = 0.025$	ns
	PN7	12.01 ± 0.16	12.40 ± 0.23	12.55 ± 0.12	13.01 ± 0.21	$P = 0.029$	$P = 0.004$	ns

Table 2. Cross-fostering body and kidney weights for male and female offspring at postnatal day 7 (PN7) (n=8-11 litters/group). Data were split by Sex and a one-way ANOVA with Tukey's post-hoc testing provided the main Treatment effect within each sex. Data presented as the mean ± SEM, where 'ns' is not significant. Significant differences between cross-foster groups are indicated by different letters ($P < 0.05$), for example 'b' is different to 'c' but not 'bc'.

	Pup -on- Mother	Control -on- Control	Control -on- Restricted	Restricted -on- Control	Restricted -on- Restricted	Treatment
Body weight	Male	10.00 ± 0.24^b	9.34 ± 0.19^b	7.47 ± 0.34^a	6.61 ± 0.39^a	$P = 0.0001$
(g)	Female	9.84 ± 0.19^b	8.88 ± 0.22^b	7.41 ± 0.38^a	6.80 ± 0.36^a	$P = 0.0001$
Kidney weight	Male	118.9 ± 3.1^c	111.8 ± 2.1^{bc}	101.1 ± 4.0^b	87.4 ± 3.8^a	$P = 0.0001$
(mg)	Female	121.5 ± 2.3^b	114.9 ± 3.4^b	100.0 ± 4.9^a	89.3 ± 3.2^a	$P = 0.0001$
Kidney/body weight	Male	11.89 ± 0.11^a	11.99 ± 0.16^a	13.59 ± 0.42^b	13.37 ± 0.43^b	$P = 0.0001$
(mg/g)	Female	12.34 ± 0.11	12.98 ± 0.34	13.51 ± 0.17	13.29 ± 0.47	ns

Table 3. Relative kidney mRNA abundance at embryonic day 20 (E20) in Control and Restricted fetuses (male and female) of genes regulating renal growth, nephrogenesis and leptin signalling (n=4-8/group, with n=1 representing 1 pup from 1 litter). Data were analysed with a two-way ANOVA to determine differences between Treatments and Sexes, with a Tukey's post-hoc test used to determine where interactions lie. Data presented as arbitrary units with the mean ± SEM, where 'ns' is not significant. Significant differences between Control and Restricted offspring are indicated by an asterisk (*$P < 0.05$).

	Male		Female		Treatment	Sex	Interaction
	Control	Restricted	Control	Restricted			
Branching Morphogenesis (n=4-7/group)							
Ret	1.00 ± 0.42	0.91 ± 0.42	0.39 ± 0.18	2.50 ± 0.63*	$P = 0.020$	ns	$P = 0.031$
Gdnf	1.00 ± 0.20	1.37 ± 0.51	1.01 ± 0.11	1.87 ± 0.68	ns	ns	ns
Tgfb1	1.00 ± 0.43	0.95 ± 0.45	1.00± 0.33	1.62 ± 0.62	ns	ns	ns
Wnt11	1.00 ± 0.31	0.88 ± 0.29	1.07 ± 0.12	1.87 ± 0.36	ns	ns	ns
Gfra1	1.00 ± 0.16	0.80 ± 0.11	1.27 ± 0.28	1.34 ± 0.28	ns	ns	ns
Angiogenesis (n=5-7/group)							
Vegfa	1.00 ± 0.21	1.16 ± 0.12	0.80 ± 0.21	2.21 ± 0.68	$P = 0.046$	ns	ns
Flt1	1.00 ± 0.54	1.35 ± 0.38	0.46 ± 0.23	2.85 ± 0.72	$P = 0.013$	ns	ns
Kdr	1.00 ± 0.41	0.77 ± 0.15	0.99 ± 0.41	1.42 ± 0.32	ns	ns	ns
Apoptosis (n=4-7/group)							

Bcl2	1.00 ± 0.33	1.01 ± 0.30	0.40± 0.12	2.09 ± 0.51[*]	P = 0.022	ns	P = 0.023
Bax	1.00 ± 0.25	1.72 ± 0.59	0.91± 0.29	3.96 ± 1.07	P = 0.016	ns	ns
Leptin Receptor (n=6-8/group)							
ObR	1.00 ± 0.28	0.50 ± 0.16	0.38 ± 0.04	0.57 ± 0.25	ns	ns	ns
Jak2	1.00 ± 0.03	1.00 ± 0.10	0.99 ± 0.11	0.76 ± 0.08	ns	ns	ns
Stat3	1.00 ± 0.08	0.88 ± 0.10	0.87 ± 0.04	0.85 ± 0.08	ns	ns	ns
Stat5a	1.00 ± 0.11	1.05 ± 0.09	1.23 ± 0.06	0.83 ± 0.11	ns	ns	P = 0.035
Socs3	1.00 ± 0.12	1.13 ± 0.10	1.16 ± 0.12	0.94 ± 0.11	ns	ns	ns
Megalin (n=6-8/group)							
Megalin	1.00 ± 0.10	1.12 ± 0.13	0.89 ± 0.06	0.83 ± 0.11	ns	ns	ns
Pi3k	1.00 ± 0.15	0.93 ± 0.08	0.91 ± 0.14	0.71 ± 0.06	ns	ns	ns
mTOR	1.00 ± 0.03	1.07 ± 0.08	1.00 ± 0.09	1.17 ± 0.14	ns	ns	ns
Akt3	1.00 ± 0.08	0.85 ± 0.09	0.88 ± 0.08	1.07 ± 0.11	ns	ns	ns
Ampka	1.00 ± 0.03	1.00 ± 0.07	1.02 ± 0.11	0.73 ± 0.08	ns	ns	ns
Ampkb	1.00 ± 0.13	1.08 ± 0.12	0.93 ± 0.10	1.03 ± 0.10	ns	ns	ns

Legends to Figures

Figure 1: Abundance of renal branching morphogenesis markers in early postnatal life. *Ret* (a), Gdnf mRNA abundance (b) and relative protein expression (c) at postnatal day (PN) 7 in the developmental study (n=6-10/group, with n=1 representing 1 pup from 1 litter). Gene data in the developmental study are analysed with a two-way ANOVA reporting differences between Treatments and Sexes, with protein data analysed with a Student's t-test. Control (open bars) and Restricted (black bars) male and female offspring. *Ret* (d) and *Gdnf* (e) mRNA abundance in male (left) and female (right) cross-fostered offspring (n=4-8/group). Cross-fostering data were split by Sex and a one-way ANOVA provided the main Treatment effect within each sex. Data are expressed as mean ± SEM, where 'ns' is not significant. Significant differences between Control and Restricted offspring are indicated by an asterisk (*P < 0.05).

Figure 2: Abundance of renal angiogenesis markers in early postnatal life. *Flt1* (a), *Kdr* (b), Vegfa mRNA abundance (c) and relative protein expression (d) at postnatal day (PN) 7 in the developmental study (n=7-10/group, with n=1 representing 1 pup from 1 litter). Gene data in the developmental study are analysed with a two-way ANOVA reporting differences between Treatments and Sexes. Control (open bars) and Restricted (black bars) male and female offspring. *Flt1* (e) and *Kdr* (f) and *Vegfa* (g) mRNA abundance in male (left) and female (right) cross-fostered offspring (n=6-8/group). Cross-fostering data were split by Sex and a one-way ANOVA with Student–Newman–Keuls post-hoc testing providing the main Treatment effect within each sex. Data are expressed as mean ± SEM, where 'ns' is not significant. Significant differences across cross-fostering groups are indicated with different letters, for example 'a' is different to 'b', but not to 'ab' (P < 0.05).

Figure 3: Abundance of renal apoptotic markers in early postnatal life. *Trp53* (a) and *Casp3* (b) mRNA abundance at postnatal day (PN) 7 in the developmental study (n=8-10/group, with n=1 representing 1 pup from 1 litter). Cleaved caspase 3 immunohistochemical straining in representative kidney sections of Control male (c) and Restricted (d) male PN7 offspring from the developmental study (inserts are negative control sections with a 100μm scale bar). Gene data in the developmental study were analysed with a two-way ANOVA reporting differences between Treatments and Sexes. Control (open bars) and Restricted (black bars) male and female offspring. *Trp53* (e) and *Casp3* (f) mRNA abundance in male (left) and female (right) cross-fostered offspring (n=6-8/group). Cross-fostering data were initially analysed with a two-way ANOVA to determine any sex-specific differences. Data were then split by Sex and a one-way ANOVA with Student–Newman–Keuls post-hoc testing providing the main Treatment effect within each sex. Data are expressed as mean ± SEM, where 'ns' is not significant. Differences between Males and Females are indicated with a hashtag (#$P < 0.05$).

Figure 4: Abundance of renal leptin signalling targets in early postnatal life. *Stat3* (a), *Megalin* (b) and *Pi3k* (c) mRNA abundance at postnatal days (PN) 7 in the developmental study (n=6-7/group, with n=1 representing 1 pup from 1 litter). Gene data in the developmental study are analysed with a two-way ANOVA reporting differences between Treatments and Sexes, with a Tukey's post-hoc test used to identify where an interaction lies. Control (open bars) and Restricted (black bars) male and female offspring. *Stat3* (d), *Megalin* (e) and *Pi3k* (f) abundance in male (left) and female (right) cross-fostered offspring (n=5-8/group). Data were initially analysed with a two-way ANOVA to determine any sex-specific differences, with a Student's t-test (interaction) to determine differences between sexes. Data were then split by Sex and a one-way ANOVA with Student–Newman–Keuls post-hoc testing providing the main Treatment effect within each sex. Data are expressed as mean ± SEM, where 'ns' is not significant. Significant differences between Control and Restricted offspring

are indicated by an asterisk (*$P < 0.05$), differences across cross-fostering groups are indicated with different letters, for example 'a' is different to 'b' ($P < 0.05$), and differences between Males and Females are indicated with a hashtag (#$P < 0.05$).

Figure 5: Nephrogenic zone analysis in early postnatal life. Representative sections of kidneys from *Control-on-Control* (a), *Control-on-Restricted* (b), *Restricted-on-Control* (c) and *Restricted-on-Restricted* (d) male offspring. Scale bar represents 100μm. Nephrogenic zone rating (e) and nephrogenic zone width (f) in male (left) and female (right) cross-fostered offspring (n=6-8/group, with n=1 representing 1 pup from 1 litter). Cross-fostering data were split by Sex and a one-way ANOVA with Student–Newman–Keuls post-hoc testing providing the main Treatment effect within each sex. Data are expressed as mean ± SEM, where 'ns' is not significant. Significant differences across treatment groups are indicated with different letters, for example 'a' is different to 'b', but not to 'ab' ($P < 0.05$).
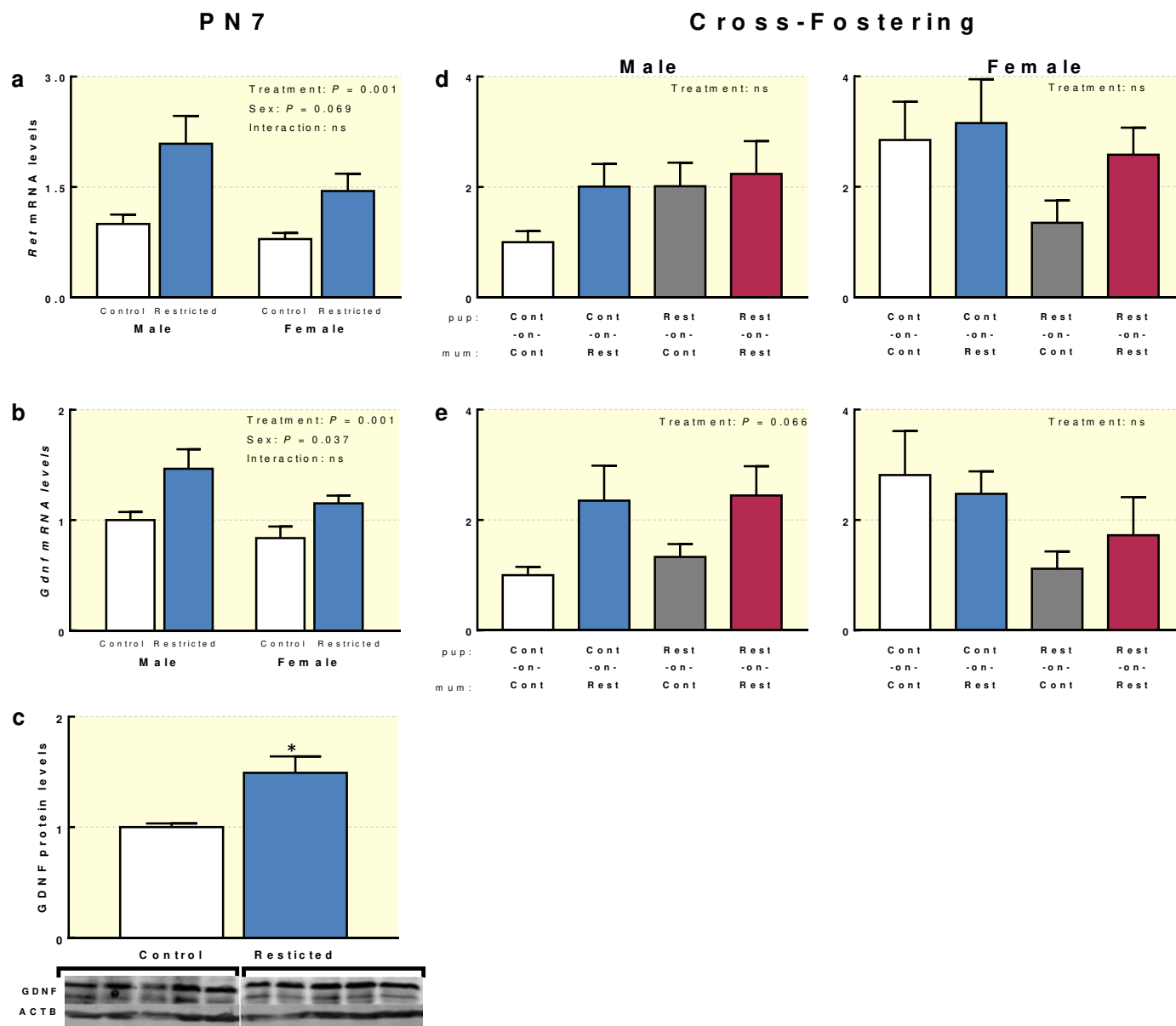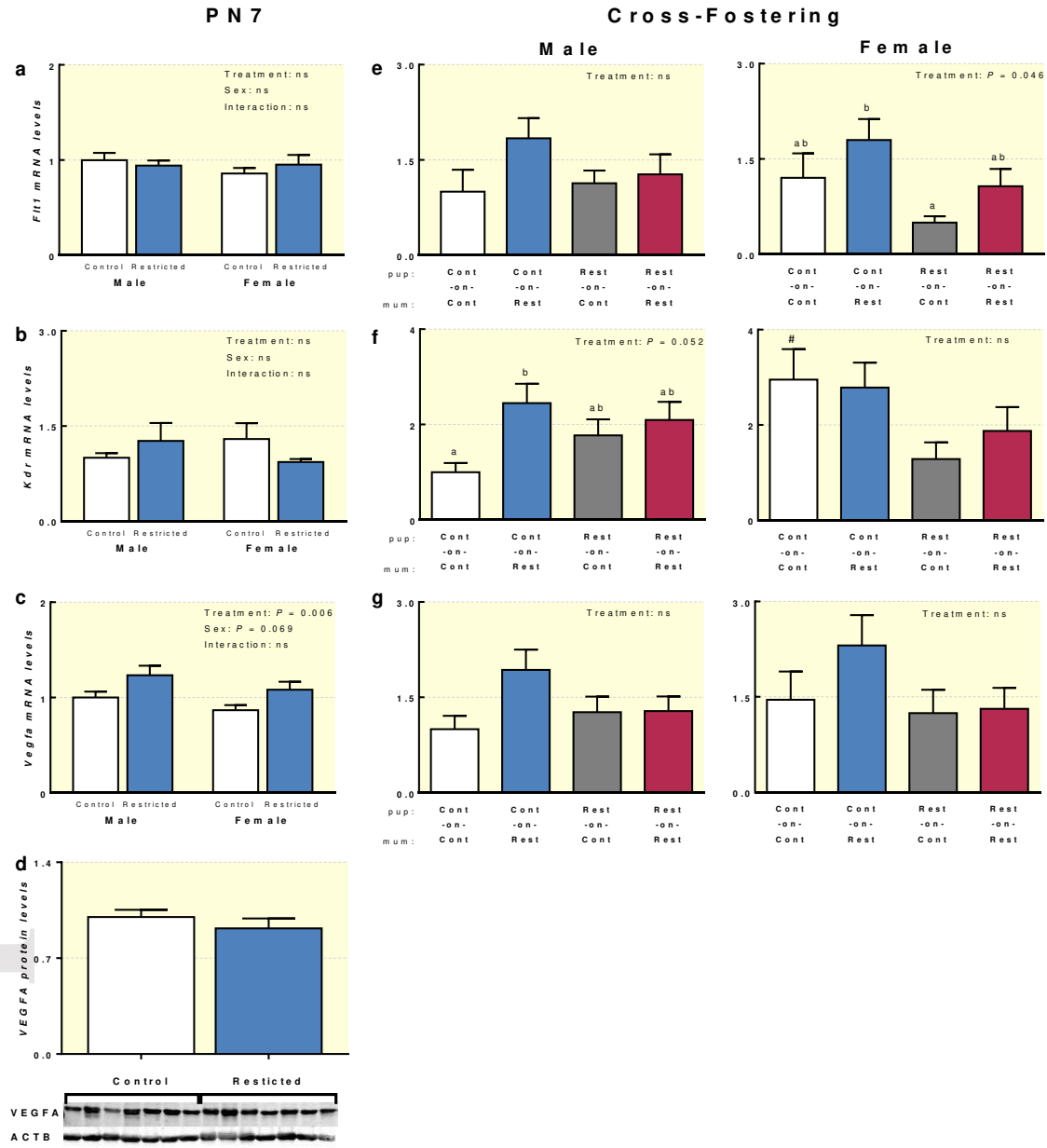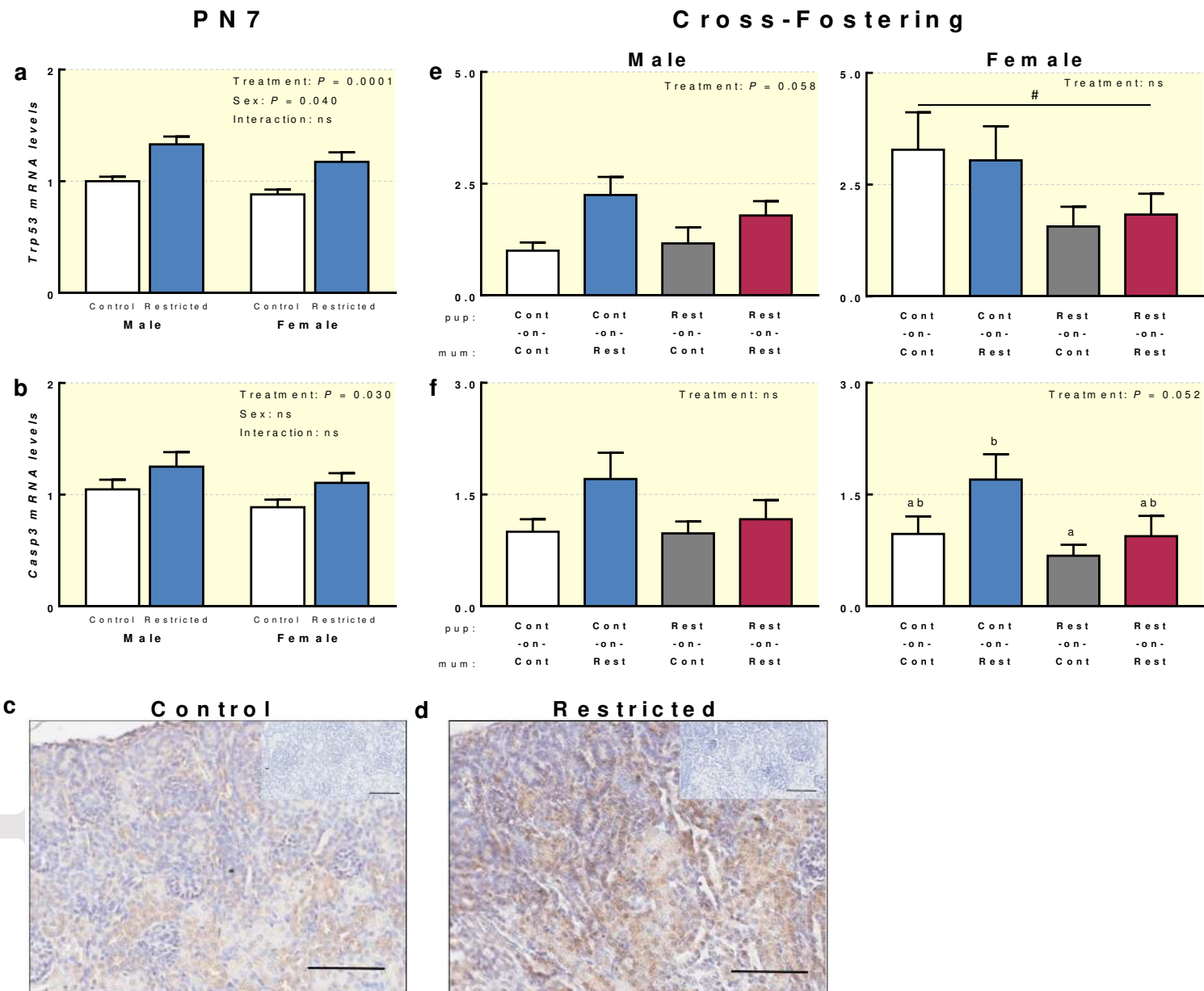
PN7

Cross-Fostering

Figure 1

Figure 2

PN7

Cross-Fostering

Figure 3

P N 7

Cross-Fostering

Figure 4

Figure 5