EphB Signalling in Rat Prostate Development

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Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is part of it being concurrently submitted in candidature for any other degree.

G R Ashley

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Abstract

In male mammals, the formation of the prostate gland is driven by androgens and involves cell-cell signalling between mesenchymal and epithelial cells. Gene profiling studies of prostate mesenchyme, using serial analysis of gene expression (SAGE), identified many transcripts that encode potential regulators of prostate development. The studies identified transcripts expressed in the ventral mesenchymal pad (VMP), a subset of the prostate mesenchyme known to express key growth factors and to regulate prostate organ development. These candidate mRNAs were used in a whole mount in-situ hybridisation (WISH) screen to identify those showing mesenchyme specific expression. The transcripts selected for WISH analysis were placed in three groups. The first group of transcripts were identified as enriched in the VMP based upon statistical analysis of their SAGE tag count. The second group of transcripts shared a SAGE tag count that was not statistically significant, and were a control for the first group. The third group encompassed transcripts that encoded either secretory or transmembrane proteins that were likely mediators of cell-cell communication. From 194 candidates, 30 were analysed by WISH and 13 were identified as mesenchymal. The tyrosine kinase receptor, EphB3, was selected from the WISH analysis and its role in prostate development was examined.

EphB signalling has been characterised as a chemotactic guidance cue in neuronal development and has also been implicated in organogenesis of the kidney, lung and colon. The Eph tyrosine kinase family is the largest of its type and is divided into two classes of receptor, EphA and EphB. The EphB family has five receptors (EphB1-4, B6) and three ligands (EphrinB1-3) in mammals. The EphrinB ligands are transmembrane proteins.

PCR analysis was used to examine the expression of the EphB and EphrinB transcripts in the developing rat prostate. The PCR analysis showed that mRNAs for the EphB2 and EphB3 receptors, and the EphrinB1 and EphrinB2 ligands, were highly expressed in the

rat prostate compared with the other EphB and EphrinB family members. The PCR analysis did not establish whether EphB receptors or EphrinB ligands were expressed in epithelia, mesenchyme or both. The EphB2 and EphB3 receptors, and the EphrinB1 and EphrinB2 ligands, were further characterised by WISH, quantitative real-time PCR and immunohistochemical analysis during prostate development. At both the mRNA and protein levels, EphB3 and EphrinB1 were expressed in a restricted area of the prostate mesenchyme, in close association with the developing epithelial buds. The EphB3 and EphrinB1 transcripts were detected by the SAGE analysis, suggesting that they were expressed in the mesenchyme. The EphB2 and EphrinB2 transcripts were not detected by the SAGE analysis, suggesting that they were expressed in the epithelium. The EphB2 receptor and EphrinB2 ligand were predominantly expressed in the developing epithelial buds, as shown by immunohistochemical analysis. The SAGE analysis of VMP mesenchyme identified EphB3 and EphrinB1 but not EphB2 and EphrinB2. This was consistent with their expression in mesenchyme or epithelium respectively.

The addition of EphB2-Fc and EphB3-Fc to *in vitro* organ cultures of neonatal prostates, acting as a ligand trap, decreased prostate growth. The addition of EphrinB1-Fc and EphrinB2-Fc ligands increased prostate organ size. The addition of EphrinB1-Fc and EphrinB2-Fc produced a significant increase in the mesenchymal and epithelial cell proliferation rates. This increase in cell proliferation in response to EphrinB1-Fc and EphrinB2-Fc was consistent with the observed increase in prostate organ size. The addition of EphB2-Fc and EphB3-Fc produced no significant increase in the mesenchymal and epithelial cell proliferation rates. This lack of a significant increase in cell proliferation in response to EphB2-Fc and EphB3-Fc was consistent with the observed decrease in prostate organ size. These findings suggest a role for EphB signalling in the regulation of prostate growth.

The addition of either EphB-Fc or EphrinB-Fc proteins to *in vitro* organ cultures resulted in a decrease epithelial branching morphogenesis. Larger epithelial buds were observed in organs treated with EphrinB1-Fc and EphrinB2-Fc, when compared to control organs. No visible change in the size of the epithelial buds was observed in response to EphB-Fc

treatment. Furthermore, p63 and Smooth Muscle Actin immunohistochemical analysis of EphrinB1-Fc and EphrinB2-Fc treated organs showed larger epithelial buds, and proliferation analysis showed greater epithelial cell proliferation in EphrinB-Fc treated organs. The increased size of each epithelial bud may be caused by the decreased epithelial branching and the increased epithelial proliferation rate, in response to the addition of EphrinB-Fc proteins. These findings suggest a role for EphB-EphrinB signalling in the regulation of prostate epithelial branching.

Collectively, we report the first reported functional link between EphB signalling and prostate development. EphB-EphrinB signalling may act as a novel juxtacrine or autocrine signal within the mesenchyme or as a novel paracrine signalling mechanism during prostate organogenesis.

Abbreviations

AP	alkaline phosphatase or anterior prostate
AR	androgen receptor
bp	base pairs
BCIP	5-bromo-4-chloro-3-indolylphosphate toluidine salt
BrdU	5-bromo-2-deoxyuridine
^0C	degrees Celsius
cDNA	copy DNA
CO_2	carbon dioxide
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
ddH_20	double distilled water
DIG	Digoxigenin
DHT	dihydrotestosterone
DLP	dorsal-lateral prostate
DMEM	Dulbecco's Modified Eagle media
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphates
DP	dorsal prostate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene diaminetetra-acetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
EphB	erythropoietin-producing hepatocellular B
FCS	foetal calf serum

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FSH	follicular stimulating hormone
GnRH	Gonadotrophin-Releasing Hormone
GPCR	G protein coupled receptor
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HCl	Hydrochorlic acid
IgG	immunoglobulin
Kb	kilobases
KCl	potassium chloride
LB	luria broth
LH	luteinizing hormone
MAPK	Mitogen-Activated-Protein-Kinase
MCS	multiple cloning site
mRNA	message ribonucleic acid
NBT	nitro-blue tetrazolium chloride
NTP	Nucleoside 5'-Triphosphate
NTMT	NaCl, Tris, $MgCl_2$, Triton X-100
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween 20
PCR	polymerase chain reaction

PKA	protein kinase A
PKC	protein kinase C
PLCγ	Phospholipase C gamma
PS-1	Prostate stromal cells
PSA	Prostate specific antigen
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
SM	smooth muscle
SSC	solution of sodium citrate
SV	seminal vesicle
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TBS	tris buffer saline
TBST	tris-buffer saline tween 20
Tfm	testicular feminized mouse strain
TGFα	transforming growth factor alpha
TGFbeta	transforming growth factor beta
UGE	urogenital epithelium
UGM	urogenital mesenchyme
UGS	urogenital stroma
UGT	urogenital tract
UR	urethra

URSC	urogenital stromal cells
UV	ultraviolet
v/v	volume to volume ratio
VMP	ventral mesenchymal pad
VMPC	ventral mesenchymal pad cells
VP	ventral prostate
VSU	Ventral mesenchymal pad, Smooth muscle, Urethral epithelium
WISH	wholemount in-situ hybridisation
w/v	weight to volume ratio

List of Figures and Tables

1 Literature Review

1.1 Introduction

The prostate is a male sex-accessory gland found in mammals and its function is the secretion of seminal fluid components (Price 1961). The prostate gland is located at the base of the bladder, surrounding the urethra. Prostate development is reliant on two key events, exposure to androgens and the molecular interactions between the mesenchyme and epithelium (Cunha and Chung 1981). In the rat, androgens bind to the Androgen Receptor (AR) expressed in an area of condensed mesenchyme called the Ventral Mesenchymal Pad (VMP) (Timms et al., 1994; 1995). This initiates a series of interactions between the mesenchyme and the epithelium that regulates the growth of epithelial buds into the mesenchyme and subsequent branching morphogenesis. The solid epithelial cords differentiate and form a secretory lumen that secretes components of the seminal fluid (Sugimura, Cunha et al. 1986). The epithelium plays an essential role in differentiating the mesenchyme of the developing prostate into the smooth muscle of a fully formed prostate (Hayward, Haughney et al. 1998). Without androgen exposure the prostate does not develop. In females, androgen levels are low and the prostate does not develop despite the presence of the VMP.

There were two main aims of this thesis. Firstly, to identify mRNAs with prostatic mesenchyme specific expression. This expression analysis would be followed by a characterisation study for one or two of these mesenchymal transcripts in terms of prostate development. The EphB3 receptor was enriched in the prostatic mesenchyme. This receptor and a number of its family members have been characterised in terms of expression and distribution, at both the RNA and protein level, during rat prostate development. Additionally, by utilising an organ culture system and recombinant proteins, an insight into the function of the EphB protein family during rat prostate development has been obtained.

This chapter is divided into two sections. Prostate development will be examined in depth including prostatic structure, function, androgens, mesenchymal-epithelial interactions and the molecules involved in this. Following this, the EphrinB ligand and EphB receptor tyrosine kinase family will be discussed. This will include their discovery, modes of action, and roles in other biological systems and disease.

1.2 Structure and Function of the Adult Prostate

The prostate gland is found only in male mammals and its function is to secrete components of the seminal fluid. Prostatic glands in adult male humans are compact glands, approximately the size of a walnut, found at the base of the bladder. The prostate gland surrounds the urethra and the terminal end of the ejaculatory duct. The structure of the human prostate is divided up into three distinct zones. These are termed the peripheral zone, the transition zone and the central zone (McNeal 1968). A diagram of the adult human prostate can be seen in Figure 1.1A. In contrast, the adult rodent prostate has a lobular structure. The regions of the rodent prostate include the anterior, dorsal, lateral, and ventral lobes. The dorsal and lateral lobes are often considered as one (dorsolateral). A representation of the adult rat prostate can be seen in Figure 1.1B.

The human and rodent prostates are similar in that both undergo branching morphogenesis and function to secrete seminal proteins (Sugimura, Cunha et al. 1986). Additionally, the rat dorsolateral lobe has been described as homologous to the human peripheral zone. However, there is no straightforward comparison between the zones of the human prostate and the rodent prostatic lobes (Abate-Shen and Shen 2000).

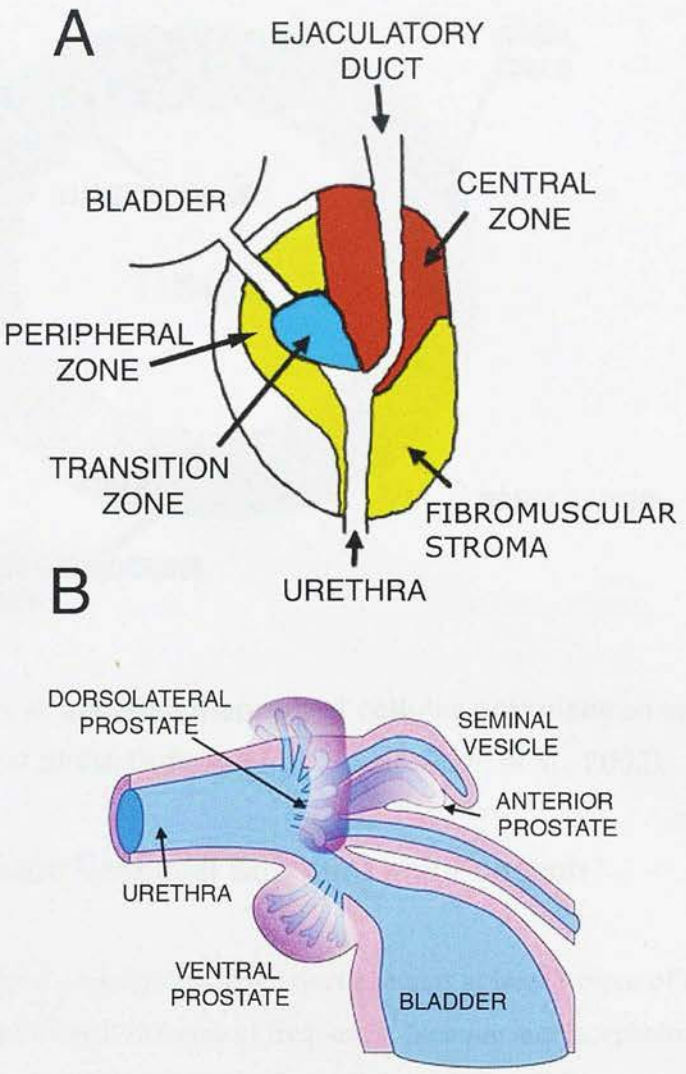

Figure 1.1. Diagrams of (A) the human prostate and (B) the rat prostate. (Adapted from McNeal 1968 and Thomson 2001, respectively)

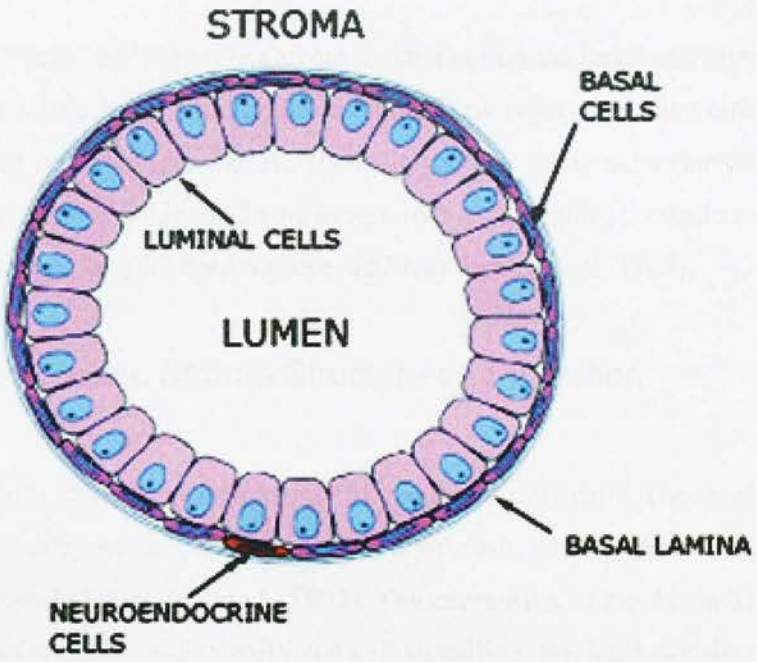

Figure 1.2. Labelled diagram of cellular organisation within prostatic epithelial ducts (Adapted from Abate-Shen et al., 2000).

Human Prostate Epithelial Structure and Function

The fully developed prostatic epithelial duct contains at least 3 types of epithelial cell. Each cell type is different in terms of frequency, function and morphology (Aumuller, 1989). The lumen is lined with the largest cell type, the luminal secretory epithelial cell. This is the cell that is responsible for producing secretory proteins that form part of the seminal fluid. At the molecular level, the luminal cells are distinguished by their specific expression of cytokeratins 8 and 18. Morphologically, the luminal cells are tall and columnar. The basal epithelial cells form a relatively continuous layer around the luminal cells. The basal cells are much smaller and express cytokeratins 5, 14 and 19. Basal cells are not known to function as secretory cells (Bui and Reiter, 1998). Additionally, there is a basal lamina that separates the epithelial and stromal compartments.

Neuroendocrine cells are rare and are located within the basal cell layer. Neuroendocrine, basal and luminal cells originate from a common endodermal pluripotent stem cell (Huss et al., 2004). In rodents, neuroendocrine cells secrete a number of neuropeptides and may have a role in regulating prostatic secretions through paracrine signalling (di Sant'Agnese, de Mesy Jensen et al. 1985).

Human Prostate Stroma Structure and Function

The prostatic stroma consists mainly of smooth muscle cells. The mesenchymal fibroblasts differentiate, under epithelial regulation, into myofibroblasts and eventually smooth muscle cells (Frid et al., 1992). The expression of the AR in SM cells is critical to its function in stromal to epithelial cell signalling. Studies have shown that smooth muscle cells in the prostate are AR positive in contrast to the AR heterogeneous fibroblasts (Shapiro et al., 1996). The main function of the prostate stroma in development is to regulate the growth and differentiation of the epithelium. In adult prostate, except from the structural function, the role of the stroma is to maintain the differentiated state of the secretory epithelial glands (Hayward et al., 1996).

1.3 Rat Prostate Development

The major changes that occur during rat prostate development are summarised in Table 1.1. The prostate gland originates from the endodermal lineage, and develops from the urogenital sinus (UGS). The UGS develops from cloaca. During mid-gestation in rodents, the cloaca is split by the urorectal septum resulting in the UGS. The UGS is the developmental source of the bladder, the prostatic urethra and bulbo-uretheral gland in males, and the urethra and lower vagina in females (Staack, Donjacour et al. 2003). The UGS is comprised of the urogenital mesenchyme (UGM) and the urogenital epithelium (UGE). Correct development of the prostate is dependent on testosterone and molecular interactions between the UGM and UGE (Cunha and Chung 1981).

The first urogenital structure can be observed at approximately at e14.5 in rats. Solid cords of epithelium from the UGE penetrate the UGM at e18.5 (Hayward, Baskin et al. 1996). The homeobox transcription factor *Nkx3.1* has been shown to be the first molecular marker of prostatic induction. In the mouse, *Nkx3.1* expression is suggested to identify urethral epithelium that will become prostate. Furthermore, *Nkx3.1* expression is far more localised at the tips of budding epithelium during postnatal development (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997). The transcription factor *FoxA2* is exclusively expressed in epithelial buds in the very early stages of murine prostate development. Therefore, it may be used as a prostate budding initiation marker similar to *Nkx3.1*. *FoxA2* may be involved in regulating prostate bud formation in the UGS based on its embryonic expression pattern (Mirosevich et al., 2005). Another member of the *FoxA* family, *FoxA1*, is widely expressed in the developing epithelium and has a functional role in prostatic morphogenesis (Gao et al., 2005). Another transcription factor that has been shown to be an early marker of prostate epithelial buds in the rat is *Sox9* (Thomsen et al., 2008).

Mesenchymal genes also have an influence in the process of rodent prostate development. A number of signalling molecules in the UGM have been examined in early rodent prostate development. The extracellular protein BMP4 appears to be a negative regulator of epithelial branching in the UGM. The expression of BMP4 is localised to the peri-epithelial mesenchyme after bud growth has begun (Lamm et al., 2001). Another protein secreted from the UGM, Noggin, a BMP4 antagonist, has been reported to have a role in the epithelial patterning of the ventral prostate (Cook et al., 2007). More recently, the Wnt5a protein has been shown to be expressed in the mesenchyme during branching morphogenesis in the rat prostate. Wnt5a inhibits epithelial branching and decreases Shh expression in early development. Androgens decrease Wnt5a expression during early development. Collectively, these observations suggest Wnt5a is a mesenchymal regulator of early rat prostate development (Huang et al., 2009).

The androgen sensitivity of the UGM is essential for rat prostate development. Androgens from the fetal testes bind to AR expressed in the UGM causing mesenchyme-epithelial interactions that initiates the UGE to bud into the UGM (Cunha and Lung, 1978). These epithelial buds grow out into highly branched epithelial cords and subsequently canalise into ducts. This complete process occurs in a proximal to distal fashion from the urethra outwards to the ductal tips (Sugimura, Cunha et al. 1986). The paracrine interactions between the branching UGE and the UGM are critical for prostatic development. An important hypothesis in the field of prostate development is the 'andromedin' hypothesis. This states that androgens acting on the UGM regulate the expression of mesenchymal paracrine factors that, in turn, affect epithelial growth and prostatic bud formation. Classic examples of potential andromedins are FGF7 and FGF10 (Yan et al., 1992, Lu et al., 1999). The FGF7 promotor has been demonstrated as androgen responsive in LNCaP (Fasciana et al., 1996) and prostate organ cultures (Thomson et al., 1997). FGF10 promotes prostate organogenesis through its role as a stromal derived paracrine factor but there is controversy over its potential function as an andromedin (Thomson and Cunha., 1999). Androgens may have a role in epithelial differentiation. Epithelial development results in a secretory luminal layer and a nonsecretory basal layer. These two layers exhibit distinct cytokeratin phenotypes once differentiation has occurred (Hayward, Haughney et al. 1998).

Timepoint	Tissue	Developmental Process	Androgen level
E15.5	UGS	Undifferentiated	2 days exposure
E16.5	UGS	Undifferentiated	3 days exposure
E17.5	UGS	Prostate buds	High
E18.5	UGS	Branching morphogenesis	High
Day 7	DLP, VP, AP	Branching morphogenesis	Low
Day 30	DLP, VP, AP	Puberty	Very High
Day 90	DLP, VP, AP	Adult	Very High

Table 1.1 Summary of the major changes during rat prostate development (Adapted from Pritchard and Nelson., 2008).

1.4 Endocrinology of Rat Prostate Development

Development of the prostate is principally driven by androgens. The primary function of androgens is to induce and regulate the male secondary sex characteristics. They are characterised as C_{19} steroids. The Leydig cells, part of the testes, are responsible for producing the main type of androgen, testosterone. Testosterone production is part of a much larger interaction of hormones and endocrine glands termed the hypothalamic-pituitary-gonadal axis (Figure 1.3). The Leydig cells are prompted to produce testosterone via Luteinising Hormone (LH) exposure. LH is secreted from the pituitary gland in response to Gonadatrophin Releasing Hormone (GnRH). GnRH is synthesised in the hypothalamus. Testosterone has a negative feedback influence on the pituitary gland, inhibiting LH production.

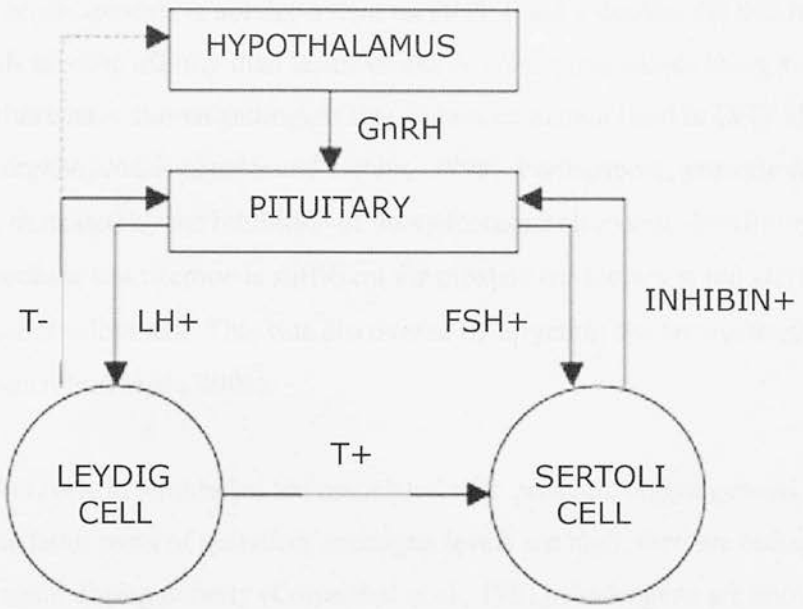

Figure 1.3. The Hypothalamic-Pituitary-Gonadal Axis Gonadotrophin Releasing Hormone (GnRH) is released from the hypothalamus and acts on the pituitary gland. Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) are released from the pituitary and act on the Leydig cells and Sertoli cells respectively. The Sertoli cells are stimulated to release Androgen Binding Protein. The testosterone produced by the Leydig cells provides a negative feedback signal to the pituitary and stimulates sperm production by the Sertoli cells. Additionally, the Sertoli cells produce Inhibin that has a negative feedback effect on the pituitary gland ('Regulation of FGF10 in Prostate Development' , Darren Tomlinson PhD thesis., 2002).

Most of the testosterone present in the bloodstream is bound to albumin and other steroid binding proteins. For example, the endocytic protein megalin has been suggested to have a role in aiding testosterone entry into prostatic epithelial cells (Hammes et al., 2005). Only the small proportion of testosterone in the blood that is free is able to enter the cell. This free testosterone is therefore the biologically active form. Once inside the cell, the enzyme 5α-reductase, which has two isoforms, 1 and 2, converts most of the testosterone to dihydrotestosterone (DHT). DHT has great potency as an androgen and binds to the AR at an affinity that is five times higher than testosterone (Wilbert et al., 1983).

Prostate organogenesis is not dependent on DHT 1 and 2 despite the fact it has a much higher AR binding affinity than testosterone. *In vitro* experiments using anterior prostate organ culture have shown androgens that cannot be metabolised to DHT also promote prostate organogenesis (Foster and Cunha., 1999). Furthermore, prostate development is not fully truncated by the inhibition of 5α-reductase 2 (Imperato-McGinley et al., 1992). This is because testosterone is sufficient for prostate development but DHT contributes to prostate development. This was discovered by targeting the 5α -reductase genes in mice (Mahendroo et al., 2001).

Androgen levels in circulation are associated with prostatic organogenesis. In the rat, during the latter parts of gestation, androgen levels are high, then are reduced post-birth and rise again during puberty (Corpechot et al., 1981). Androgens are known to be responsible for stimulating the growth of epithelial buds into the inductive mesenchyme of e17.5 embryos (Lasnitzki and Mizuno., 1980). Furthermore, androgenic action on the prostatic mesenchyme is essential for inducing correct epithelial proliferation (Shannon and Cunha., 1983; Takeda et al., 1985).

The VMP is present in e18.5 male and female rat embryos. This indicates VMP formation is independent of androgens. Androgen exposure of the female urogenital tract *in vitro* leads to prostate budding (Takeda et al., 1986). The VMP may induce epithelial branching through paracrine mechanisms. Many stromal derived factors, that are influential on the prostatic epithelium, have been identified. These include Insulin–like growth factors I and II (Cohen et al., 1991), FGF7 (Yan et al., 1992), FGF10 (Nakano et al., 1999), and SFRP1 (Joesting et al., 2008). These molecules are stromal paracrine effectors but there is still debate whether some of them are regulated by androgens (Thomson, 2001). Androgens have also been shown to decrease the expression of inhibitory stromal proteins such as TGFbeta (Itoh et al 1998; Tomlinson et al., 2004). This is true of another inhibitory protein, BMP4 (Lamm et al., 2001) that is downregulated by androgens in the rat ventral prostate (Pu et al., 2007).

The smooth muscle (SM) layer is a barrier between the urethra and the VMP in rat urogenital tracts. There is evidence that androgens regulate prostatic development via the SM layer. At e20.5, the SM layer develops between the urethral epithelium and the VMP, but SM layer development does not occur in males (Thomson et al., 2002). In female reproductive tracts cultures, testosterone exposure results in a 2.4-fold reduction in thickness of the SM layer (Chrisman and Thomson., 2006). These observations led to the formation of the SM hypothesis - The SM layer acts as a barrier to epithelial branching into the VMP. Androgens may be responsible for the thinning of the SM layer in the male, allowing prostate development to occur.

The SM layer forms at e20 in rats. This time point is after the start of prostatic induction in terms of epithelial budding. This suggests the SM layer inhibits paracrine mechanisms involved in the prostate organogenesis, but not the initial induction. This hypothesis is supported by the association of the SM layer synthesis with the closure of a "programming window" (Cunha and Lung., 1978; Welsh et al 2007, 2008). The SM layer provides a newly proposed novel mechanism for androgens to regulate prostate organogenesis and offers an alternative to the andromedin hypothesis described earlier.

Oestrogens are the other major steroid hormone group that play a role in prostate development. The levels of circulating oestrogens in the male are much less than the female (Belanger et al., 1994; Ferrini and Barrett-Connor, 1998). However, a key enzyme involved in oestrogen synthesis, cytochrome P450 aromatase, is expressed in prostatic tissues (Matzkin and Soloway., 1992; Negri-Cesi et al., 1999; Ellem et al., 2004). The prostate of Aromotase knockout mouse (ArKO) mice significantly reduced aromatase activity, which leads to prostate hypertrophy. These observations suggest correct oestrogen metabolism is crucial for normal prostate development (McPherson et al., 2006; Jarred et al., 2002; Simpson et al., 2004).

The two oestrogen receptors, ERα and ERβ, are expressed in the rodent prostate. In early prostate development, ERα is detectable in the mesenchyme prior to being widely

expressed in the developing gland. ERα expression is suppressed by androgens in later development. ERβ is not expressed in early prostatic development (Cunha et al., 1987; Prins et al 2001). The expression patterns are reversed in adulthood (Makela et al., 2000; Pelletier et al., 2000; Omoto et al 2005). The presence of both ER types during prostate development combined with the observations from the ArKO mice indicate that oestrogen signalling, like androgens, have a role in rodent prostate development.

1.4 Stromal-Epithelial Interactions

Mesenchymal-Epithelial Interactions during Development

The AR is essential for correct mesenchymal-epithelial interactions prostate development. During the initial stage of prostatic development, AR expression is confined to the mesenchyme. After the start of epithelial branching morphogenesis, AR expression can be found in the epithelium. All this suggests that prostate budding from the epithelium is mediated by androgen induced factors from the mesenchyme. The role of the AR in epithelial tissues is to regulate prostate control, by inhibiting epithelial proliferation and secretory activities, in the adult prostate gland (Wu et al., 2007).

The interactions between the mesenchyme and epithelium are essential for prostate development. The initial signalling stems from the UGM and these paracrine signals produce prostatic bud induction. The epithelium reciprocates the signalling by mediating the conversion of inductive mesenchyme into fibroblasts and smooth muscle. The spontaneous mutation that produced the androgen insensitive *tfm* (testicular feminised) mouse (Lyon and Hawkes., 1970) allowed elegant tissue recombination experiments to be conducted. By recombining wild type prostatic mesenchyme with *tfm* epithelium it was shown that the AR in mesenchyme, not epithelial, that was crucial for prostatic development (Cunha et al., 1980).

The mesenchyme and epithelium tissues used for these recombination experiments were isolated using dissection and enzymatic digestion from the UGS and the other tissues. *Tfm* mice have no prostate so their tissues are very useful when used in conjunction with wild type tissues for recombination experiments (Figure 1.4). When *tfm* mesenchyme was recombined with either *tfm* or wild type epithelium, no prostatic structures developed. However, when wild type mesenchyme was recombined with either *tfm* or wild type epithelium, prostatic structures formed. This shows that it is AR in mesenchyme, not epithelia, that is crucial for prostate development (Cunha and Chung., 1981). When *tfm* epithelium was recombined with wild type mesenchyme, no secretions were observed from the luminal epithelium, although prostatic structures formed. This indicates that androgens induce luminal secretions via the epithelial AR later in development. The overall conclusions from the work by Cunha and colleagues are supported by AR expression studies (Hayward et al., 1996; Cooke et al., 1991) and investigations into androgen binding sites using autoradiographic techniques (Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985).

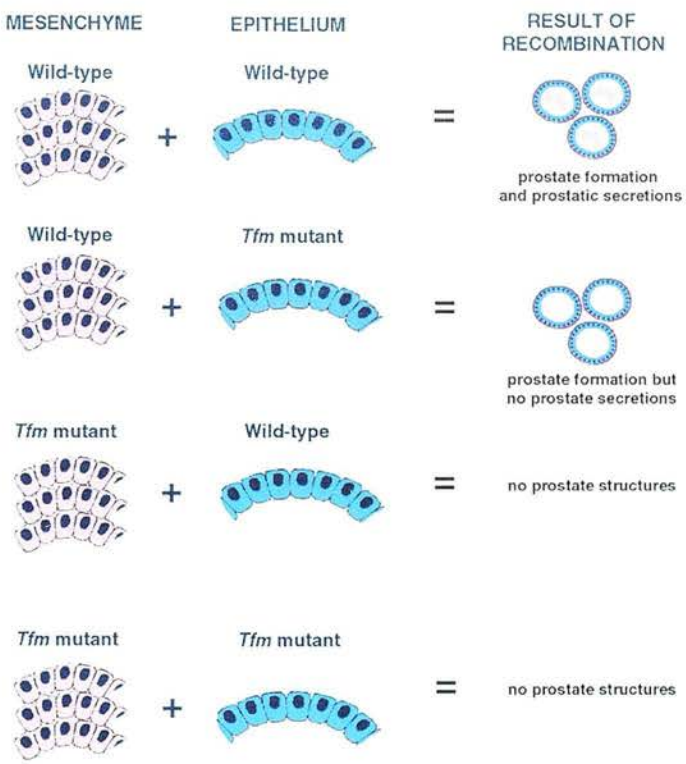

Figure 1.4 Results summary of tissue recombination experiments. These were performed by Cunha et al 1978, utilising *tfm* and wild type mice tissues.

Stromal-Epithelial Interactions during Adulthood

The stromal and epithelial cells that comprise the adult prostate interact in a state of homeostasis that regulates adult prostate function. These reciprocal interactions are the maintenance of SM differentiation by the epithelium and the SM in turn sustains the adult epithelial structure (Cunha et al., 1994; Cunha et al., 1996; Cunha et al., 2004; Hayward et al., 1998; Jin et al., 2004). One pathway that does not have a role in prostatic homeostasis is the mixed lineage kinases (Gao and Issacs., 2001). Much is unknown about the role of stromal-epithelial interactions in prostatic homeostasis in adults. However, the signalling pathways that have been characterised as having a role in

prostate development may also have a role in prostatic maintenance, such as the FGF family (Cunha et al., 1996). FGF10 plays a vital role in prostate development (Thomson and Cunha., 1999; Donjacour et al., 2003; Huang et al., 2005). FGF10 in the mesenchyme and FGFR2 in the epithelium represent one of the best-known mechanisms of stromal-epithelial interactions during prostate development.

Aberrant paracrine signalling in the prostate may have a role in prostate carcinomas (Hayward et al., 1997; Olumi et al., 1999; Barclay et al., 2005). The Notch signalling pathway has been characterised as having a role in prostate development, primarily through Notch1 expression in the epithelium and Notch1 knockouts result in epithelial hyperplasia (Wang et al., 2006). Furthermore, Notch2 signalling has a role in the prostate stroma development (Orr et al., 2008). As Notch signalling has a role in both development and disease of the prostate, it is likely it is needed for prostatic homeostasis.

The Secreted Frizzled-related Proteins (SFRPs) modulate of Wnt signaling through direct interaction with the Wnt proteins. SFRP1 has low expression in the adult rat prostate (Joesting et al., 2005). Overexpression of SFRP1 in the adult prostate of mice increases epithelial proliferation but decreases secretory gene expression (Joesting et al., 2008). These observations suggest that Wnt signaling regulation has a role in the adult prostate. Members of the TGFβ beta family are expressed in the adult rat prostate. PCR analysis has revealed that TGFβ-13 is expressed in the epithelium and stroma and TGFβ-1 is stroma in the adult rat prostate (Hayward et al., 1998). In aging rat prostates tissue, all three TGFβ genes are expressed (Zhao et al., 2002). TGFβ-1 regulates prostate development via FGF10 (Tomlinson et al., 2004). TGFβ signaling may have a regulatory role in the maintenance of the adult prostate. BMP signaling represents another signaling system that may have a role in the adult prostate. BMP2, 3, 4 and 5 mRNA have been detected in adult rat and human prostatic tissue. BMP4 was predominant in the human prostate (Harris et al., 1994). BMP7 is expressed in the adult prostate but this expression is lower in developing and neoplastic prostate tissue

(Masuda et al., 2004). These observations suggest BMP signaling, likely involving BMP7, is involved in the maintenance of the adult prostate.

This thesis addresses the role of the EphB-EphrinB signalling pathway in prostate development. The next section will discuss the known expression and functions of EphB-EphrinB signalling in the development of other tissues and the role that EphB-EphrinB signalling pathway may play in disease.

1.6 Signalling families involved in rat prostate development

A number of signalling families have been reported as having a role in the development of the rat prostate, such as the FGF family. The FGF10 ligand is expressed in the prostatic mesenchyme whereas its receptor, FGFR2b, is restricted to the epithelium. This paracrine signalling mechanism is essential for normal prostatic organogenesis in an AR independent fashion (Thomson and Cunha., 1999; Lin et al., 2007). Conversely, an increase in FGF10 mRNA has been reported in prostatic fibroblasts treated with testosterone (Lu et al., 1999).

The Sonic Hedgehog (*Shh*) ligand is expressed in the epithelial buds of the developing rat prostate. Conversely, the receptor Patched (*Ptc*) is localised to the surrounding mesenchyme. Shh has been shown to regulate prostatic growth and epithelial differentiation (Freestone et al., 2003). It has been demonstrated that *Shh* signalling pathway is not required for prostatic induction because budding occurs in *Shh* mutant mice. Furthermore, after treating wild type urogential explants with the *Shh* inhibitor cyclopamine, prostatic epithelial budding was not truncated. However, the cyclopamine treatment did have an effect on epithelial duct morphology (Wang, Shou et al. 2004). These results suggest that *Shh* signalling does not have a role in prostate induction or budding. However, *Shh* signalling may have a function in prostatic duct patterning (Berman et al., 2004).

The Notch1 receptor is expressed in the basal epithelial cells of rat prostatic ducts. Additionally, it may function to define the progenitor cells of the epithelial lineage. Using a transgenic cell ablation approach, prostate branching morphogenesis and differentiation have been reported to be dependent on Notch1 positive epithelial cells (Wang et al., 2004, 2006). The Notch2 receptor and the Delta-like 1 (Dlk1) ligand have been reported as expressed in the peri-epithelial mesenchyme of the developing rat prostate (Orr et al., 2009). This suggests a role for Notch2 signalling within the prostatic mesenchyme in either an autocrine or juxtacrine manner.

Another signalling family reported to play a role in rat prostate development is the Wnt family. Wang et al (2008) show that Wnt signalling modulates prostatic epithelial branching morphogenesis and luminal epithelial cell differentiation. Rat ventral prostates were cultured in the presence of either the Wnt stimulating factor, Wnt3a, or an inhibitory factor, DKK1. Addition of Wnt3a increased cell proliferation and reduced luminal cell differentiation. Conversely, addition of DKK1 increased cell differentiation and decreased cell proliferation. These results suggest a regulatory role for Wnt signalling in rat prostate development. Furthermore, the gene Axin2 is a Wnt pathway transcriptional target. Axin2 was highly expressed in the developing prostate but down regulated in the adult prostate, further suggesting a developmental role for Wnt signalling.

Rat prostate development involves a plethora of different signalling pathways that have roles to play in the regulation of prostatic organogenesis. It is likely other known signalling pathways also have roles in the development of the prostate.

The prostate gland is an organ with highly specialized functional attributes that serves to enhance the fertility of mammalian species. Much of the information pertaining to normal and pathological conditions affecting the prostate has been obtained through extensive developmental, biochemical and genetic analyses of rodent species. Although important insights can be obtained through detailed anatomical and histological

assessments of mouse and rat models, further mechanistic explanations are greatly aided through studies of gene and protein expression. RESULTS: In this article we characterize the repertoire of genes expressed in the normal developing mouse prostate through the analysis of 50,562 expressed sequence tags derived from 14 mouse prostate cDNA libraries. Sequence assemblies and annotations identified 15,009 unique transcriptional units of which more than 600 represent high quality assemblies without corresponding annotations in public gene expression databases. Quantitative analyses demonstrate distinct anatomical and developmental partitioning of prostate gene expression. This finding may assist in the interpretation of comparative studies between human and mouse and guide the development of new transgenic murine disease models. The identification of several novel genes is reported, including a new member of the beta-defensin gene family with prostate-restricted expression. CONCLUSIONS: These findings suggest a potential role for the prostate as a defensive barrier for entry of pathogens into the genitourinary tract and, further, serve to emphasize the utility of the continued evaluation of transcriptomes from a diverse repertoire of tissues and cell types.

A common approach used to identify genes involved in prostate development is gene profiling. Expressed Sequence Tags (ESTs) have been used to profile transcription within the mouse prostate (Abbott et al., 2003). Zhang et al (2005) used a serial analysis of gene expression (SAGE) approach that identified components of the Wnt signalling family in early prostate development. More recently, SAGE has been used to identify mRNAs localised to the VMP (Vanpoucke et al; 2007). Gene profiling is a quick method of identifying candidate genes and pathways involved in prostate development.

1.7 The Eph-Ephrin Signalling Family

Discovery and Organisation

The Eph receptor family is the largest receptor tyrosine kinase (RTK) family. To date, 16 receptors (14 found in mammals) and 9 ligands (8 in mammals) have been identified. The first member of the Eph family was identified and cloned from an Erytropoietin Producing Hepatocellular (Eph) carcinoma cell line (Hirai et al., 1987). Various members of the Eph receptor and Ephrin ligand family were soon discovered and were all given different names. In 1997, the Eph Nomenclature Committee standardised the terminology (summarised in Table 1.2) The Eph family is separated into two classes, EphA and EphB. The main differences between the two classes are their ligand specificity. EphA receptors bind preferentially to EphrinA, and EphB receptors to EphrinB ligands (Himanen and Nikolov., 2003). However, some crosstalk can occur between the A and B classes. For example, receptor EphA4 and ligand EphrinA5 can interact with EphrinB2 and EphB2, respectively (Bouzioukh et al., 2007; (Himanen, Chumley et al. 2004).

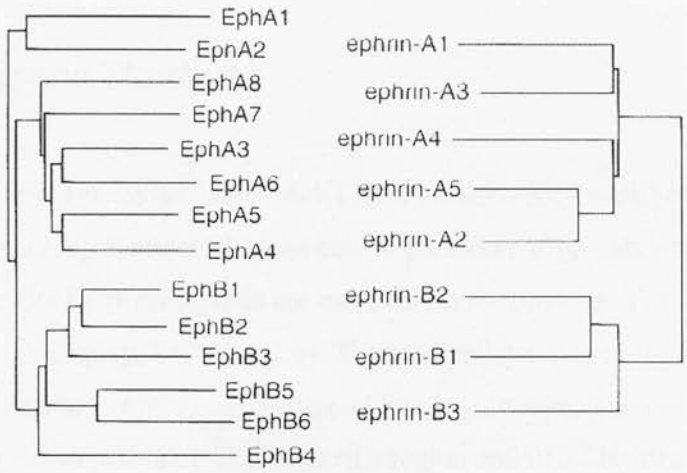

Figure 1.5. Sequence Homology Trees for Eph receptors and Ephrin ligands (Adapted from Eph Nomenclature Committee, 1997)

Receptors		Ligands	
New Name	**Preivous Name**	**New Name**	**Previous Name**
EphA1	Eph, Esk	**EprhinA1**	B61, LERK-1, EFL-1
EphA2	Eck, Myk2, Sek2	**EphrinA2**	ELF-1, Cek7-L, LERK-6
EphA3	Cek4, Mek4, Hek, Tyro4, Hek4	**EprhinA3**	Ehk1-L, EFL-2, LERK-3
EphA4	Sek, Sek1, Cek8, Hek8, Tyro1	**EphrinA4**	LERK-4; EFL-4
EphA5	Ehk1, Bsk, Cek7, Hek7, Rek7	**EphrinA5**	AL-1, RAGS; LERK-7, EFL-5
EphA6	Ehk2, Hek12		
EphA7	Mdk1, Hek11, Ehk3, Ebk, Cek11		
EphA8	Eek, Hek3		
EphB1	Elk, Cek6, Net, Hek6	**EphrinB1**	LERK-2, Elk-L, EFL-3, Cek5-L, STRA-1
EphB2	Cek5, Nuk, Erk, Qek5, Tyro5, Sek3, Hek5, Drt	**EphrinB2**	Htk-L, ELF-2, LERK-5, NLERK-1
EphB3	Cek10, Hek2, Mdk5, Tyro6, Sek4	**EphrinB3**	NLERK-2, Elk-L3, EFL-6, ELF-3, LERK-8
EphB4	Htk, Myk1, Tyro11, Mdk2		
EphB5	Cek9, Hek9		
EphB6	Mep		

Table 1.2 Nomenclature for the Eph Receptor and Ephrin Families.
(Adapted from Eph Nomenclature Committee, 1997)

Eph-Ephrin Structure

The Ephrin ligands are membrane bound proteins and consist of two groups. The EphrinA group is attached to the cell surface via a glycosylphosphotidylinositol (GPI) anchor. The EphrinB ligands are transmembrane proteins. The structure of the Eph receptor is displayed (Figure 1.6). The extracellular domain has many components. The globular domain functions as a ligand-binding domain. The rest of the extracellular domain is comprised of a cysteine rich region and two fibronectin type III repeats.

The intracellular component of the receptor has four main sections. The juxtamembrane contains two conserved tyrosine residues. The other three components are a protein tyrosine kinase domain, a sterile α-motif (SAM) and a PDZ binding domain. The structure of the SAM domain suggests dimers and oligomers can form. Additionally, a consensus binding sequence exists in the PDZ binding domain that includes a hydrophobic residue at the very carboxyl terminus (Labrador et al., 1997; Himanen et al., 1998; Toth et al., 2001; Himanen et al., 2001).

EphA receptors bind EphrinA ligands and EphB receptors bind EphrinB ligands. However, exceptions have emerged in recent years. For example, EphrinA5 can activate the EphB2 receptor (Himanen et al., 2004) and EphA3 and EphrinB2 can interact *in vitro* (Cerretti et al., 1995; Lackmann et al., 1997). Therefore there is the potential for signalling of A and B class ligands through both A and B class receptors.

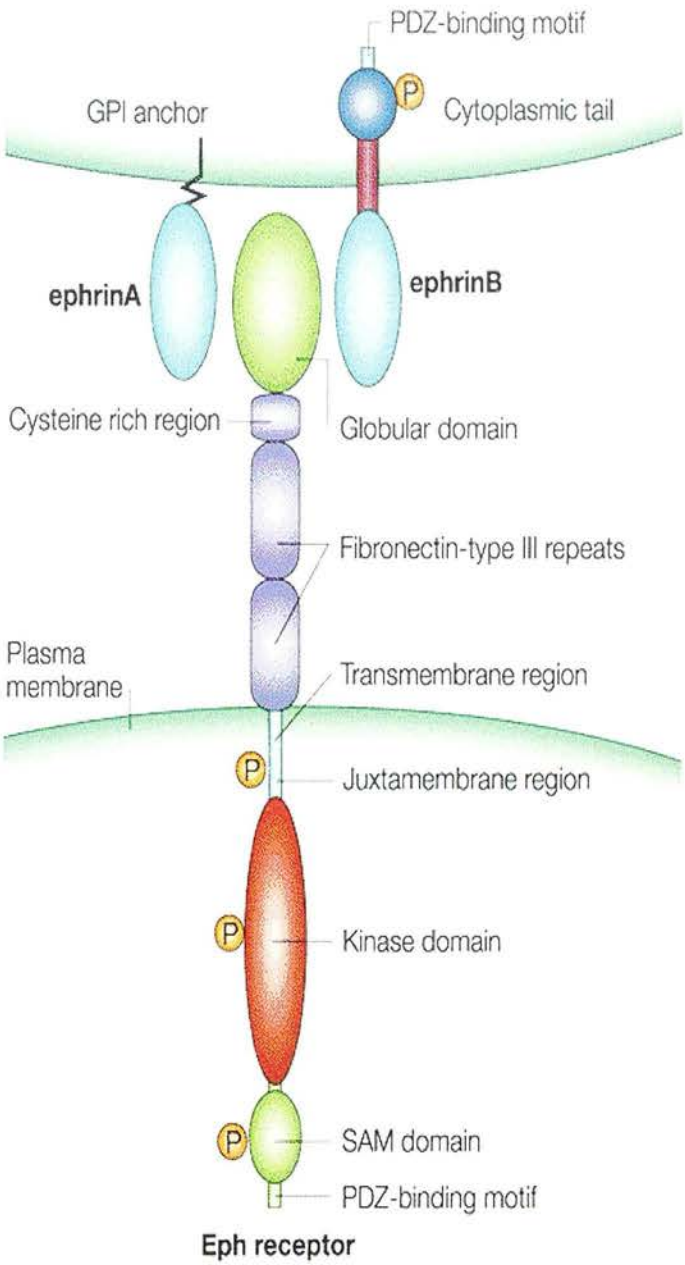

Figure 1.6. General features of Eph receptors and ephrins. (Taken from Kullander and Klein., 2002).

Eph-Ephrin Functions

The role of Eph as a chemotactic signal has been studied most extensive in neural and vascular development (Wilkinson et al., 2001; Adams et al., 2002; Poliakov et al., 2004). Other functions that the Eph family have roles in include cell-cell adhesion, modulating the actin cytoskeleton, intracellular junctions, and cell shape and movement (Pasquele 2005; Egea and Klein., 2007; Himanen et al., 2007;). Eph function has also been implicated in developmental contexts. More specifically, these include EphB2 and EphrinB2 in urorectal development (Dravis et al., 2004), EphB2 and EphrinB2 in kidney homeostasis (Ogawa et al., 2006, Hashimoto et al., 2007) and EphrinB2 in lung development (Wilkinson et al., 2008). These examples are relevant to prostate development because the prostate is a urogenital organ that uses branching morphogenesis as a developmental mechanism, similar to the kidney and lung. Additionally, these examples relate to members of the EphB-EphrinB signalling family that are the focus of this thesis.

A unique feature of the EphB receptors and the EphrinB ligands are their capability to produce bidirectional signals that affect both receptor expressing cells and ligand expressing cells (Mellitzer et al., 1999). EphB receptor 'forward' signalling mediates the phosphorylation of other proteins in addition to the autophosphorylation of the receptor. These processes are dependent on the EphB receptor's intracellular tyrosine kinase domain. The intracellular region of the EphrinB ligand provides the tyrosine phosphorylation that is essential for EphrinB ligand 'reverse' signalling. This is a signal from a receptor expressing cell influencing downstream gene transcription in a ligand expressing cell (Egea and Klein., 2007). This reverse signalling commences in EphrinB clusters via their phosphorylation by Src kinases (Holland et al., 1996). These supply docking sites for adaptor proteins such as Growth Factor Receptor-Bound protein 4 (Grb4), for the initiation of signalling mechanisms that regulate the actin cytoskeleton

(Cowan et al., 2001). The diverse functions and signal transduction mechanisms of Eph signalling will be discussed later.

EphB signalling and Prostate Cancer

There is evidence to suggest that EphB signalling has a role to play in prostate carcinogenesis. It has been shown that EphB2 may have a critical role in prostatic cell migration during cancer. The combined techniques of nonsense-mediated RNA decay microarrays and array-based comparative genomic hybridisation revealed, a biallelic nonsense mutation in the EphB2 gene of the DU145 prostatic cancer cell line. This Q723X amino acid change truncated the EphB2 receptor at the kinase domain leading to functional redundancy. Moreover, approximately 8% of primary and metastatic prostate cancers examined possessed the truncating mutation. Functional evidence for EphB2 having a role in prostatic cell migration was shown when DU145, which lacks functional EphB2, was transfected with wild type EphB2. This transfection resulted in over 80% clonogenic growth reduction (Huusko et al., 2004). This data suggests EphB2 may be a tumour suppressor gene in prostate cancer.

Fox et al (2006) conducted an expression profile of EphB receptors and EphrinB ligands in normal prostate, primary prostatic tumours and invasive prostatic tumours. EphB2 was found to have increased expression in the normal prostate and primary prostatic tumours compared to the invasive prostatic cell lines. This reinforces other evidence implicating EphB2 as a prostatic tumour suppressor gene (Hussko et al., 2004). The EphB3 receptor is expressed in normal prostate and invasive cell lines but not primary cancer samples. These observations suggest EphB3 maybe a tumour suppressor because it is downregulated in the primary cancer samples. However, as it expressed in the invasive cell lines, this suggestion may seem unlikely. The EphrinB1 and EphrinB2 ligands were expressed in normal prostate, primary tumours and invasive cell lines at similar levels (Fox et al., 2006). The metalloprotein, Azurin, is structurally similar to the EphrinB2 ligand and has been shown to preferentially enter cancer cells, and induce

apoptosis. Azurin possesses a high binding affinity for the EphB2 receptor. In DU145 cells with functional EphB2, azurin shows a cytotoxic effect whereas as the EphrinB2 ligand increased cell proliferation (Chaudhari et al., 2006). Collectively, this evidence suggests EphB signalling has a role in prostate cancer progression that has yet to be fully characterised.

1.8 EphB Signal Transduction and Intracellular Signalling

Eph receptors and Ephrin ligands on adjacent cells can dock with their amino-terminal domain to create dimers in addition to higher order clusters at the cell-cell interface (Himanen et al., 2001). These higher order structures induced by Ephrin ligand binding autophosphorylates the Eph receptor cytoplasmic tyrosines (Kalo et al., 1999). This autophosphorylation weakens the inhibitory interactions that exist between the kinase and juxtamembrane domains of the Eph receptor. This boosts kinase activity and produces docking sites for downstream signalling proteins with SH2 domains (Binns et al., 2000). However, some essential protein interactions occur in the absence of Eph receptor autophosphorylation. These encompass interactions with PDZ domain containing-proteins and many guanine nucleotide exchange factors (GEFs) for Rho family proteins (Kullander and Klein., 2002).

Eph receptors have both kinase dependent and kinase independent functions mediated through relatively unknown mechanisms. This section will review the current level of understanding with regard to the signal transduction and intracellular signalling of the EphB-EphrinB interactions.

EphB Signalling with Rho proteins

The major transducers of EphB forward (ligand to receptor) signalling are the small GTP-binding proteins of the Ras and Rho families (Noren and Pasquale., 2004). Activated Rho and Ras proteins bind to downstream effectors to promote pathways that regulate many cellular properties such as actin cytoskeleton organisation, cell migration and gene transcription. Two members of the Rho family are Rac1 and Cdc42. In hippocampal (forebrain) neurons EphB2 signals through these two proteins by associating with Intersectin and Kalirin, two exchange factors for Cdc42 and Rac1, respectively. Intersectin binds to the EphB2 kinase domain regardless of receptor activation (Irie et al., 2002; Penzes et al., 2003). Neural Wiskott-Aldrich syndrome protein (N-WASP) acts with EphB2 to stimulate Intersectin. N-WASP is known to interact with Arp 2/3, an actin-polymerising complex; this gives direct connection from EphB forward signalling to actin filament regulation. Intersectin2 is also known to have a downstream function from Eph forward signalling (Schmidt and Hall., 2002; Cory et al., 2002).

Another EphB associated exchange factor is Kalirin. It is mainly expressed in the nervous system and has a role in spine morphogenesis (Penzes et al., 2001). EphrinB1 can activate EphB2 receptors in immature hippocampal neurons. This provides Kalirin recruitment at EphB2 clusters and positively regulates dendritic spine morphogenesis. Kalirin only binds to the activated form of EphB2 (Penzes et al., 2003). EphrinB1 ligand exposure to hippocampal neurons increases Kalirin phosphorylation and upregulates Pak activation. Pak is a serine/threonine kinase and a downstream effector of Rac1. Another example of Rho protein activity from EphB activation comes from Moeller et al (2006). Dendritic spine morphogenesis is regulated by EphB receptors. The downstream effect of the EphB receptors is FAK (Focal Adhesion Kinase) mediated RhoA activation that contributes to actin filament formation in dendrite spines.

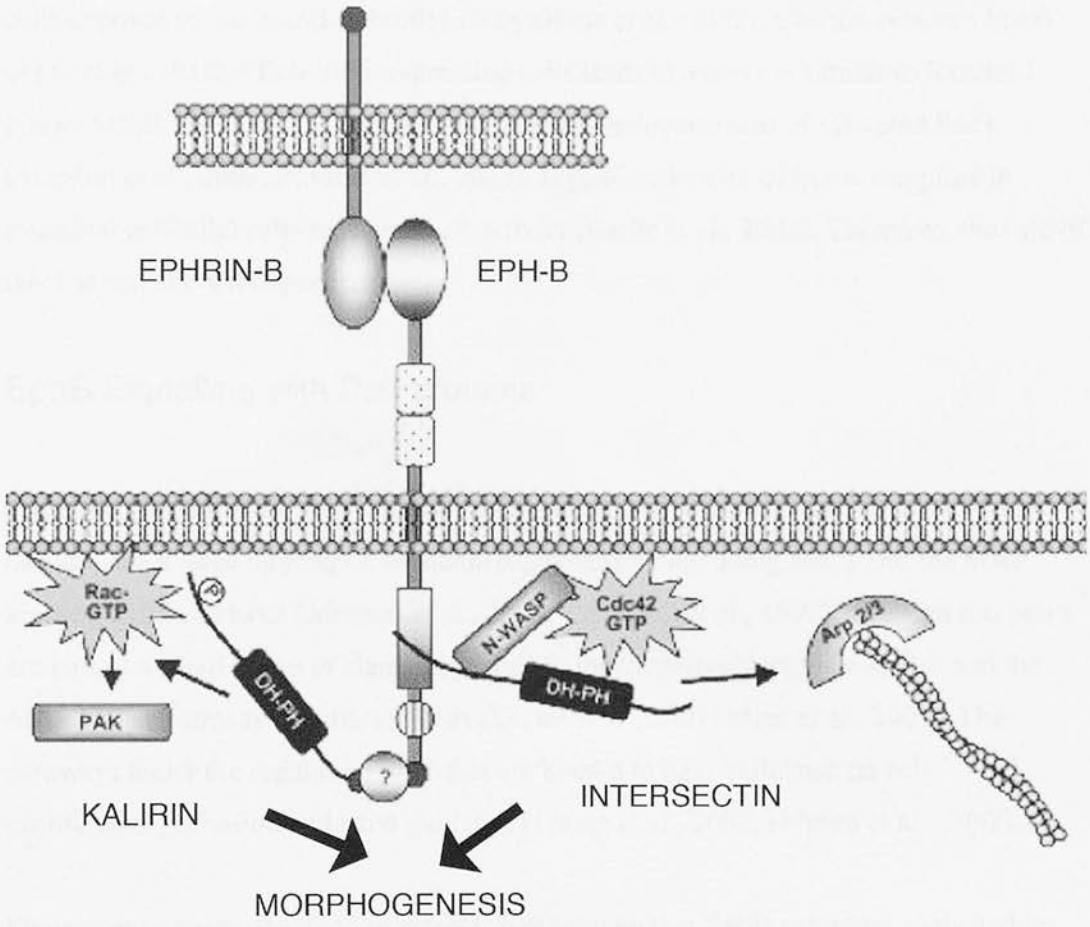

Figure 1.7 GEFs that bind to EphB receptors. EphB receptors signal to Rac1 and Cdc42 through Kalirin and Intersectin in hippocampal neurons (Adapted from Noren and Pasquale., 2004).

The Pak-interacting exchange factor, αPix, is an additional exchange factor for Rac1 and Cdc42. αPix may have a function in GTPase activation downstream of the EphB receptor (Yoshii et al., 1999). αPix is linked with activated EphB2 via an interaction with Nck, an adaptor protein (Holland et al., 1997). Pix and Pak both localise to focal adhesions, and maybe they modulate integrin activity downstream of EphB signalling.

Rac1 is needed for cellular spreading and membrane ruffling in human aortic endothelial cells exposed to the ligand EphrinB1 (Nagashima et al., 2002). Contact between EphB expressing cells and EphrinB1 expressing cells leads to a process similar to localised phagocytosis and is reliant on actin polymerisation downstream of activated Rac1 (Marston et al., 2003; Zimmer et al., 2003). Ligand activation of EphB receptors in intestinal epithelial cells reduces Rac1 activity (Batlle et al., 2002). Therefore, the role of Rac1 is cell context dependent.

EphB Signalling with Ras proteins

The main Ras protein linked with EphB signalling is H-Ras. In its active form, H-Ras can activate a large number of serine/threonine kinase including Mek1 and the MAP kinases, Erk1 and Erk2 (Johnson et al., 2002; Campbell et al., 1998). The Eph receptors are part of a small group of signalling families that negatively regulate H-Ras and the MAP kinase pathway in different cells (Elowe et al., 2001; Miao et al., 2001). The pathways under the regulation of H-Ras are known to have influence on cell proliferation, adhesion and axon guidance (Forcet et al., 2002; Hughes et al., 1997).

Fluorescent energy transfer experiments have shown that EphB receptors, activated by EphrinB1 ligand, in human aortic endothelial cells inhibit Ras activity. Ras/MAP kinase inhibition provides an explanation for the decrease in cell proliferation rate by Eph receptors (Nagashima et al., 2002). Eph receptors can also inhibit the Ras/MAP kinase pathway in hippocampal neurons (Grunwald et al., 2001).

Eph receptors can activate Ras pathways, not just inhibit them. Overexpression of EphB receptors in many cell types can activate Erk1 and Erk2 MAP kinases (Grunwald et al., 2001; Vindis et al., 2003). The EphB receptors have a role in the regulation of Ras family members other than the MAPK proteins. Integrin mediated adhesion is inhibited by EphB2 via tyrosine phosphorylation in the R-Ras effector domain (Zou et al., 1999). This phosphorylation curbs the capability of R-Ras to stimulate integrins.

SHEP1 is a candidate signalling protein to link EphB2 to R-Ras. SHEP1 can bind directly to both EphB2 and R-Ras, indicating that SHEP1 localises R-Ras at sites of EphB2 activation (Dodelet et al., 1999). Rap1, related to R-Ras, may also be linked to EphB receptors through SHEP1 though a protein complex including the adaptor protein Crk. In response to EphrinB1 stimulation, Rap1 is activated at membrane ruffles of human aortic endothelial cells. This activation is Crk dependent. This provides evidence that Rap1 interacts with EphB receptors (Nagashima et al., 2002).

To summarise, the major transducers of EphB forward (ligand to receptor) signalling are the small GTP-binding proteins of the Ras and Rho families (Noren and Pasquale., 2004). GTPases are molecular switches that flux between the inactive GDP-bound state and the active GTP-bound state (Holland et al., 1996). The utilisation of GEFs allows the exchange of GDP for GTP. This exchange is inhibited by GTPase-activating proteins (GAPs), which mediates GTP hydrolysis to GDP. To complicate the picture further, Guanine nucleotide Dissociation Inhibitors (GDIs) have many functions such as membrane translocation regulation. These GDIs additionally negatively regulate Rho proteins (Bourne et al., 1991).

EphB Signalling and G protein coupled receptors (GPCRs)

EphrinB reverser signalling can regulate downstream signals of GPCRs. PDZ-RGS3 is a GTPase activating protein for GPCRs and the PDZ domain of this protein docks constitutively with the intracellular PDZ-binding domain of the EphrinB1 ligand. Clustering of EphrinB1 occurs at the EphB2 receptor in cerebellar granule neurons. Following this, PDX-RGS3 impairs chemoattraction via Stromal Derived Factor 1 (SDF1). The chemokine SDF1 binds the CXCR4 receptor that is a GPCR. EphB receptor activation of EphrinB1 affects cell migration in the cerebellum by making the neurons insensitive to SDF1 (Lu et al., 2001). This represents an example of EphrinB reverse signalling. More recently, EphrinB1 reverse signalling has been shown to lead to

STAT3 transcription in the nucleus mediated by the tyrosine kinase Jak2 (Bong et al., 2007). STAT3 plays important roles in development and tumourigenesis. The fact that EphrinB reverse signalling is responsible for its activation suggests EphrinB signalling has more roles in development that are yet to be elucidated.

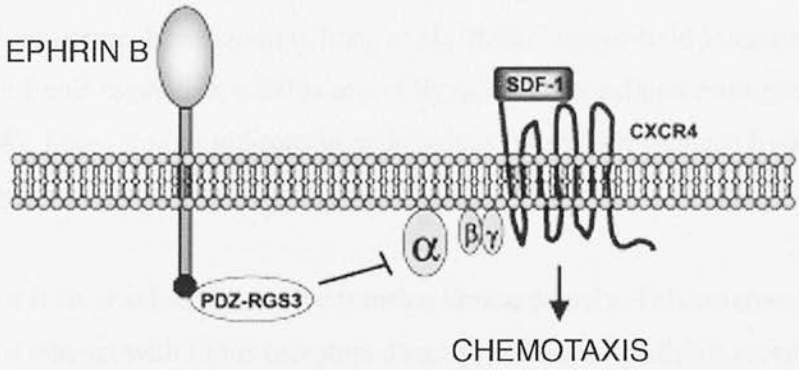

Figure 1.8 EphrinB signalling via PDZ-RGS3 inhibits chemotaxis. SDF1 binds to CXCR4 and activates intracellular signalling. PDZ-RGS3 binds constitutively to the PDZ domain of EphrinB and induces a reversal of SDF1 initiated signalling by inactivating the α subunit of the protein complex at CXCR4 (Adapted from Noren and Pasquale., 2004).

Interestingly, SDF1 and CXCR4 have been shown to have a role in prostatic tumorigenesis *in vitro* and *in vivo* via crosstalk with the TGFβ pathway. SDF1 is produced by Cancer Associated Fibroblasts (CAFs), and acts through the TGFβ-regulated CXCR4 to stimulate the serine-threonine kinase, Akt. This Akt activation occurs in epithelial cells and promotes tumourigenesis. This means the inhibitory effects of TGFβ become redundant (Ao et al., 2007). EphB receptors do appear to crosstalk with other signalling pathways.

EphB Signalling Crosstalk with Cell Surface Receptors

A large number of reports have suggested interactions between Eph-Ephrin signalling and another large family of receptor tyrosine kinase (RTK), the FGF family. In *Xenopus* embryos, activated FGF receptor (FGFR) can bind to EphrinB1 and inhibit its ability to induce blastomere dissociation (Chong et al., 2000). In eye field formation in *Xenopus*, FGF and EphB regulate eye fields access by modulating cellular movement (Moore et al., 2004). These studies indicate an antagonistic interaction between Ephrin and FGF pathways.

RYK is a RTK that has an inactive tyrosine kinase domain. This inactive RTK has been shown to interact with EphB receptors directly, and activated EphB receptors can phosphorylate the tyrosine residues of RYK in the mouse. The recruitment of AF-6, a junction protein, to EphB receptors could mediate downstream effects (Halford et al., 2000). Additionally, the same direct interaction between EphB3 and RYK has been observed in rats. RYK regulates cell migration through binding to EphB receptors during rat cortical development. It was shown that RYK binds to EphB3 in particular *in vitro* (Kamitori et al., 2005).

RYK is a receptor for Wnt proteins and an antagonistic association exists between Wnt/RYK and EphB-EphrinB signalling in retinotectal topographic mapping (Inoue et al., 2004; Lu et al., 2004; Schmitt et al., 2006). Wnt3/RYK has a function as a lateral mapping force to counterbalance the medial mapping force of EphB-EphrinB signalling in the dorsal midbrain (Schmitt et al., 2006).

EphB Signalling Crosstalk with Adhesion Proteins

There is evidence to suggest the activation of EphB-EphrinB signalling increases integrin mediated cell adhesion (Huynh-do et al. 1999; Prevost et al., 2004, 2005). Conversely, other reports show negative regulation of integrin mediated cell adhesion by EphB signalling (Zou et al., 1999). These regulatory outcomes do not seem to be connected to a particular EphB receptor/Ephrin ligand binding pair. Contrasting effects of EphB signalling on integrins is likely due to different cellular contexts. The EphB and integrin interaction is at the level of intracellular downstream signalling. The signalling molecules involved are either cytoplasmic kinases such as FAK or MAPK or small GTPases such as Rac, Rho or Ras. There is only one report that indicates a direct association between an EphB receptor and an integrin (Prevost et al., 2005).

The Immunoglobin superfamily (IgSF) of proteins are involved in many developmental processes, such as chemotactic guidance, like the EphB family. WRK-1 is a *C.elegans* IgSF protein that associates with EphB signalling (Boulin et al., 2006). The axon guidance receptor, Robo, is a member of the IgSF family and is also involved in *C.elegans* midline guidance (Dickson and Gilestro., 2006). The *C.elegans* Robo homologue is sax-3 that associates with vab-1, an Eph receptor, to regulate axon guidance and morphogenesis. Direct binding between sax-3 and vab-1 has been shown using yeast two-hybrid and GST-pull down assays (Ghenea et al., 2005). Evidence for associations between EphB and IgSFs in higher order organisms is more lacking. The EphB2 receptor has been demonstrated to bind to the IgSF L1 (Zisch et al., 1997). Additionally, neuronal growth cones exposed to endogenous L1 became insensitive to EphB2 (Suh et al., 2004).

Claudins are a key component in tight junctions that are found in the lateral membrane. Tight junctions represent a partition that limits molecular movement between epithelial

barriers (Hartstock and Nelson., 2007). Claudin-4 has been reported to bind to EphrinB1. This causes EphrinB1 tyrosine phosphorylation that has downstream implications for cellular adhesion in mammalian epithelial cells (Tanaka et al., 2005). EphrinB1 has additionally been reported to associate with Par6. This scaffold protein is required in the construction of tight junctions. The association between EphrinB1 and Par6 is inhibited by EphrinB1 cytoplasmic tyrosine phosphorylation. Furthermore, EphrinB1 competes with Cdc42 for association with Par6. This competition can result in the loss of tight junction formation (Shuk-Lee et al., 2008).

EphB Signalling Crosstalk with Cell Surface Proteases

Presenilins are highly conserved transmembrane proteins that are the active component of γ-secretase complexes (Takasugi et al., 2003). Through the action of an aspartate protease, the γ-secretase complexes cleave single span transmembrane proteins to produce a separate intracellular domain (ICD). These ICDs have been reported to function as transcriptional activators (e.g. Notch and CD44) or repressors (Jagged and N-cadherin) within the nucleus (Reviewed in Kopan and Ilagan., 2004).

EphrinB ligand ICDs have been identified as components of EphrinB reverse signalling. EphB-EphrinB2 binding has been shown to promote the sequential processing of EphrinB2 by matrix metalloprotease (MMPs) and γ-secretase to generate an EphrinB2 ICD. This novel ICD binds to Src and removes a Src repressor named Csk, permitting Src activation. EphrinB2 ICD degradation has been shown to decrease Src activation (Georgakopoulos et al., 2006). Furthermore, an EphrinB1 ICD has been reported to be involved in actin filament regulation (Tomita et al., 2006). These two examples implicate γ-secretase in EphrinB reverse signalling. Other reports have shown that the cleaving of other domains may occur in EphB forward signalling as well.

The EphB2 receptor has been shown to undergo proteolytic cleavage by two mechanisms, calcium influx and EphrinB2 ligand binding. The protease ADAM10 was identified as the enzyme for calcium mediated processing of the EphB2 receptor extracellular domain. The interactions between EphB2 and EphrinB2 gave increased extracellular domain cleavage. This was subsequently followed by γ-secretase activity and EphB2 ectodomain breakdown (Litterst et al., 2007). Proteolytic cleavage has roles in the transduction of both EphB forward and EphrinB reverse signalling.

1.9 EphB-EphrinB Functional Roles in Biological Processes

Neuronal Development and Axon Guidance

EphB-EphrinB signalling modulates a number of cell-cell contact dependent processes in both the developing (Cooke and Moens, 2002; Poliakov et al., 2004) and mature (Murai and Pasquale., 2002; Yamaguchi and Pasquale., 2004) central nervous system (CNS). A repulsive response is the main function of the EphB signalling in neuronal movement. However, an attractive cue is another role for EphB-EphrinB signalling (Holmberg and Frisen., 2002; Eberhart et al., 2004).

EphB-EphrinB signalling is extremely well documented in the nervous system. Some of the neuronal process that EphB-EphrinB interactions regulate include neural crest cell migration (Krull et al., 1997; Santiago and Erickson., 2002; Davy et al., 2004), neuroblast migration (Conover et al., 2000), inner ear axon guidance (Cowan et al., 2000; Brors et al., 2003), retinal ganglion cell axon guidance (Birgbauer et al., 2000; Mann et al., 2002; McLaughlin et al., 2003), and hippocampal axon bundling (Chen et al., 2004). In the spinal cord itself, the EphrinB ligands have been reported to have several roles. EphrinB3 has a repulsive function at the ventral midline of the developing mouse spinal cord (Imondi et al., 2000). EphrinB1 and EphrinB2 are known to repel embryonic spinal motor axons *in vitro* (Wang and Anderson., 1997). Furthermore,

EphrinB induced forward signalling is needed for murine spinal midline guidance decisions (Kadison et al., 2006).

Angiogenesis

Many of the EphB-EphrinB family members are expressed in the cardiovascular system. However, the EphB4 receptor and the EphrinB2 have been the main research focus in the field. EphB4 expression is mostly venous-specific in contrast to the expression of EphrinB2 that is largely restricted to arterial endothelial cells. Targeted inactivation of EphB4 and EphrinB2 has shown that they are crucial for angiogenic remodelling (Wang et al., 1998; Adams et al., 1999). EphrinB2 also has a critical role in blood vessel endothelial lining (Gerety and Anderson., 2002).

The Eph receptors and Ephrin ligands are involved in the regulation of common developmental systems. The Notch pathway is responsible for modulating the simultaneous EphrinB2 up regulation and EphB4 down regulation in the arterial endothelium (Heroult et al., 2006). EphrinB2 activity is also increased by exposure to Vascular Endothelial Growth Factor (VEGF) (Gale et al., 2001). Although the genetic evidence for EphB involvement in angiogenesis is substantial, the mechanism by which they function has not been elucidated. Exposure of cultured endothelial cells to EphB and EphrinB recombinant proteins stimulates branching angiogenesis (Palmer et al., 2002). This suggests forward, reverse or both signalling directions are involved in the mechanism. EphB4 and EphrinB2 have been also been associated with the development of the lymph system. It has been observed that EphB4 is expressed in the lymphatic vasculature and EphrinB2 in the endothelium of the collecting lymphatic vessels (Makinen et al., 2005). The EphB family clearly have a role to play in angiogenesis and vascular development but its mechanistic action is yet to be known.

Immunology and Immunological Development

A number of the Eph receptors and their respective ligands are expressed in immune cells, particularly during T-cell development (Gurniak and Berg., 1996). The kinase deficient EphB6 receptor is capable of signalling in T-cells, likely through an adapter protein such as Cbl or Grb2 (Luo et al., 2001). These studies show that EphB signalling is both present and functional in T-cells. EphrinB1 in particular has received much attention in the field of immunology.

EphrinB1 has been reported to stimulate T-cell proliferation. MAPK signalling is enhanced by EphrinB1-Fc exposure to cultured mice T-cells. Furthermore, EphrinB1 knockdown mice experience comprised T-cell development and function (Yu et al., 2004). More recently it has been reported that EphrinB1 mediated signalling has a crucial role in maintaining the organisation of 3D epithelial structure that supports T-cell maturation *in vivo*. Additionally, EphB-Fc and EphrinB-Fc treatments reduce the extent of cell conjugations between thymocytes and thymic epithelial cells (Alfaro et al., 2007). It is clear that EphB-EphrinB interactions play a very active role in the immune system.

Stem Cell Niche Regulation

Stem cell proliferation is partly mediated by neighbouring cells that create the stem cell niche (Mikkers and Frisen., 2005). A number of studies have implicated EphB and EphrinB proteins as functional components in the regulation of these niches. For instance, a number of gain and loss of function experiments revealed a role for EphB signalling in the intestinal stem cell niche. By using overexpression mutations in the EphrinB2 and EphB2 genes, it was concluded the enhanced EphB signalling increases intestinal progenitor cell proliferation. EphB2 and EphB3 knockout mice showed that

these kinase dependent signalling receptors stimulate cell-cycle re-entry of intestinal progenitor cells (Holmberg et al., 2006).

Another example of EphB involvement in stem cell biology comes from dental pulp stem cells (DPSCs). EphrinB1 and EphrinB3 are expressed in the tissue surrounding the DPSC while most of the EphB receptors themselves are expressed predominantly on the DPSC. The bidirectional signalling caused by the EphB-EphrinB interactions has been reported to inhibit cell spreading. The mechanism by which this was achieved was the MAPK pathway for forward signalling and Src phosphorylation for EphrinB reverse signalling. Forward signalling through the EphB receptors is also responsible for impairing DPSC migration (Stokowski et al., 2007). EphB receptors have also been shown to have *in vivo* roles in hippocampal stem/progenitor cell regulation in the forebrain through interactions with EphrinB3 (Chumley et al., 2007).

As EphB signalling has been reported to have functional roles in intestinal and dental stem cell regulation, they may have involvement regulating in stem cells in other organs.

Roles in Other Organs

The EphB-EphrinB signalling system has additionally been shown to have functions in a number of other organs such as the kidney and lung. Both the kidney and lung develop through the mechanism of branching morphogenesis like the prostate.
However it is in the adult kidney where EphrinB1 and EphB2 have putative roles regulating the cellular architecture and spatial organisation of renal tubules. These roles were elucidated in various assays utilising the EphrinB1-Fc and EphB2-Fc recombinant proteins and the EphB2 antagonistic peptide, SNEW. Through the application of a Rho kinase inhibitor, it was revealed the activation of the RhoA-ROCK intracellular pathway that modulates the EphB receptor effects on cellular morphology (Ogawa et al., 2006). RhoA proteins are known to mediate EphB downstream effects.

EphB signalling has more recently been reported as having a functional role in the development of the lung. More specifically, the ligand EphrinB2 has an essential role in postnatal alveolar development and elastic matrix integrity. Mice EphrinB2 knockouts display a near complete alveolar absence. Additionally, a mouse EphB3 knockout displays a reduced distribution of the elastic matrix component fibrillin-1 (Wilkinson et al., 2008).

EphB-EphrinB bidirectional signalling has been shown to have a role in urorectal development. The EphrinB2 ligand and the receptors EphB2 and EphB3 have cell adhesion roles that canalise the urethra and split the urinary and alimentary tracts. Mice that carry a mutation that specifically disrupts EphrinB2 reverse signalling develop hypospadia and incomplete cloacal septation. Furthermore, 25% of mice with compound EphB2/EphB3 knockouts gave a hypospadia phenotype. This data indicates that both forward and reverse EphB-EphrinB signalling is required for correct urorectal development in the mouse (Dravis et al., 2004).

EphB Signalling and Cancer

Epigenetic silencing of Eph genes and somatic mutations within their DNA sequences has been discovered in many forms of cancer. Evidence has come forward for EphB-EphrinB signalling involvement in both tumour suppression and promotion. It appears the role of EphB-EphrinB interactions depends on tumour type and cellular context (Merlos-Suarez and Batlle., 2008).

The best-studied role of EphB-EphrinB signalling in cancer is in colorectal cancer. During normal intestinal development, EphB2 and EphB3 signalling regulates the positioning of cell types along the crypt-villus axis (Batlle et al., 2002). In colorectal cancer, tumour cells that exhibit EphB receptors are unable to spread into EphrinB1 positive areas of the intestine, both *in vivo* and *in vitro*. This tumour

compartmentalization leads to tumour suppression in the colon. The mechanism is dependent on the capability of EphB signalling to maintain E-cadherin adhesion (Batlle et al., 2005; Cortina et al., 2007).

EphB signalling has been reported to play a role in breast cancer. In mammary carcinoma cells, the EphB4 receptor is known to induce an anti-oncogenic pathway consisting of the Abl tyrosine kinases and the adapter protein Crk. The EphB4 receptor can act like a tumour suppressor in a mouse xenograft model of breast cancer when stimulated by EphrinB2. The Abl-Crk pathway is likely to mediate the anti-neoplasic effects of EphB4 activation (Noren et al., 2006). Furthermore, an Abl kinase inhibitor is capable of impairing EphB4-induced tumour suppression. Collectively, this suggests the EphB4 receptor may function as a tumour suppressor gene in the context of breast cancer.

The EphB2 receptor is mutated in approximately 10% of prostate cancer tumours. Additionally, the prostate cancer cell line DU145 lacks functional EphB2. When DU145 cells are transfected with wild type EphB2, clonogenic growth is reduced (Huusko et al., 2004). This data suggest a role as a tumour suppressor gene for EphB2 and its inactivation through mutation may affect prostate cancer phenotypes. These aspects include abnormal growth, invasion and metastasis. As with EphB4 in breast cancer, EphB2 is a candidate tumour suppressor in prostatic neoplasia.

1.10 Summary

The presence of androgens and correct mesenchymal-epithelial interactions are both vital for prostate development (Cunha et al., 1983; Tenniswood., 1986). Mesenchymal-epithelial interactions are vital in the development of many other organs, for example the lung and kidney. Many different proteins and signalling pathways have been reported to be important in the regulation of prostate organogenesis (Cohen et al., 1991; Lamm et al., 2001; Donjacour et al., 2003; Pu et al., 2004; Cook et al., 2007). Despite this level of knowledge of prostate development, the picture is not complete. There are many genes and pathways that have a functional role in prostate development, which are yet to be discovered and characterised.

The EphB proteins are one of the largest families of tyrosine kinase receptors. Both the EphB receptors and their EphrinB ligands are transmembrane proteins that bind in a cell-to-cell contact dependent manner (Mellitzer et al., 1999). The Rac and Rho proteins are the major transduction intracellular signalling mechanisms for EphB-EphrinB signalling (Groeger et al., 2007). These intracellular mediators can be activated in both the receptor and ligand expressing cells through the bidirectional signalling nature of the EphB family. The downstream effects of EphB signalling include cell migration, proliferation and positioning. The downstream effects are well characterised in neural development (Wilkinson et al., 2000). However, EphB-EphrinB interactions have functions in other developmental systems such as lung and urorectal development (Wilkinson et al., 2008; Dravis et al., 2004).

Previously, a serial analysis of gene expression (SAGE) identified 219 mesenchyme-enriched transcripts (Vanpoucke et al; 2007). Mesenchyme signalling molecules are known to play a key role in prostate development. A whole mount in-situ hybridisation analysis identified 13 mRNAs restricted to the mesenchyme, including a mRNA that

encodes the EphB3 tyrosine kinase receptor. Following this, the EphrinB1 ligand was shown as expressed in the prostate mesenchyme and the EphB2 receptor and EphrinB2 ligand appeared to be epithelial specific. Functional analysis of the EphB-EphrinB signalling family *in vitro* revealed possible mechanisms by which EphB-EphrinB regulates rat prostate development.

1.11 Thesis Objectives

The objectives of this thesis were firstly to identify a number of transcripts with expression patterns restricted to the prostate mesenchyme using whole mount in-situ hybridisation (WISH). The candidate transcripts for this purpose came SAGE data examining transcript abundance in the neonatal rat prostate (Vanpoucke et al., 2007).

Following this, this project aimed to characterise a small number of the mesenchymal transcripts, along with the transcript's gene and gene product, in the context of prostate development. Expression and functional analyses was conducted using a range of different techniques including quantitative PCR, immunohistochemistry and *in vitro* organ culture.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

2.1.2 Computer Software

2.1.3 Enzymes

2.1.4 Nucleic Acids

2.1.5 Chemicals

2.1.6 Organ and Culture Media, Reagents and Equipment

2.1.7 Immunohistochemical and In-situ hybridisation reagents

2.1.8 Kits

2.1.9 Standard Solution (A-Z)

2.1.10 WISH Solutions (A-Z)

2.2 Methods

2.2.1 Wholemount in-situ hybridisation (WISH)

2.2.2 RNA probe production

2.2.3 *In Vitro* Organ Culture

2.2.4 Quantitative RT-PCR

2.2.5 Histology and Immunohistochemistry

2.1 Materials

2.1.1. Equipment

Agarose Gel electrophoresis was performed using Bio-Rad Equipment.

Nucleic acid concentrations were determined using a Nanodrop Spectrophotometer ND-1000.

Whole mount in-situ hybridisations were performed using an Invatis InsituPro VS robot and pictures taken using a Leica MZ95 microscope and camera.

PCR was performed using a PTC-200 Peltier Thermal Cycler DNA Engine from MJ Research and visualised on a Syngene Bio Imaging GeneFlash UV transilluminator.

Confocal Microscopy was performed on Zeiss LSM 510 Laser Scanning microscope.

Paraffin embedding was performed on a Leica TP1050 and tissue sections were cut using the Leica Microtome RM2135.

Histological and Immunohistological pictures were taken using an Olympus Provis AX70 microscope and a Canon EOS 30D camera.

All quantitative PCR was performed using an Applied Biosystems 7900HT Fast Real Time PCR System.

2.1.2 Computer Software

Gene sequences for PCR primer design were obtained from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and BLAST analysis was performed using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). PCR primers were designed using Invitrogen Vector NTI software. Organ culture area and distal perimeter measurements were made using NIH ImageJ software (http://rsb.info.nih.gov/ij/download.html). All organ culture, in-situ hybridisation, immunohistochemical and histological images were prepared using Adobe Photoshop CS2. All graphs and statistics were produced and performed using Graphpad Prism 5. Statistical analysis used was the unpaired student T-test a confidence value of $P=<0.05$.

2.1.3 Enzymes

Platinum Taq DNA Polymerase and Superscript II RNase H- Reverse Transcriptase were purchased from Invitrogen. *EcoR1* and *Not1* restriction enzymes were purchased from Promega. *Spe1* restriction enzyme and T3 and T7 RNA polymerases were purchased from Roche. TURBO DNA-free™ DNAse was purchased from Ambion. Proteinase K was purchased from Sigma-Aldrich.

2.1.4 Nucleic Acids

1 kb DNA Ladder was purchased from Promega. 100 mM dNTP Set (PCR Grade) was purchased from Invitrogen. DIG RNA Labelling Mix was purchased from Roche. Synthetic oligonucleotides were synthesised by MWG Biotech AG.

2.1.5 Chemicals

Recombinant RNAase Inhibitor was purchased from Promega.

Chemicals for standard solutions were obtained from Sigma-Aldrich and were of analytical grade.

2.1.6 Organ Culture Media, Reagents and Plastics

All plates and pipettes were purchased from NUNC or Falcon.

DMEM/F12+Glutamax-I organ culture media (Cat no 31331-028), Transferrin bovine-halo form (Cat 11107-018), and Antibiotic/Antimycotic Solution (Cat no 15240-62) were purchased from GIBCO/Invitrogen. Insulin solution with 25 mM HEPEs (Cat no I0516-5M0) were purchased from Sigma-Aldrich.

4 well plastic culture plates (Cat no 176740) were purchased from NUNC™. Millicell® CM culture plate inserts (Cat no PIC M03050) were purchased from Millipore.

All recombinant proteins were purchased from R&D Systems. EphB3-Fc (Cat number 432-B2-200), EphB2-Fc (Cat number 467-B2-200), EphrinB1-Fc (Cat number 473-EB-200), EphrinB2-Fc (Cat number 496-EB-200), and Mouse IgG-Fc (Cat number 4460MG).

Quantitative PCR were performed using MicroAmp Fast Optical 96-well reaction plate (Cat no. 4346906) and Optical Adhesive Film (Cat no. 4311971).

2.1.7 Immunohistochemical and In-situ hybridisation reagents

Normal Goat and Rabbit serum were supplied by Diagnostic Scotland.

Table 2.1 Primary antibodies used and their dilutions in Immunohistochemistry

Antibody	Source	Species	Specificity	IHC Dilutions	Concentration
Anti-Cytokeratin	Sigma Aldrich (Cat no C2562)	Mouse	Monoclonal	1 in 200	N/A
Anti-BrdU	Fitzgerald International, (Cat no 20BS170)	Sheep	Polyclonal	1 in 300	250µg/ml
Anti-p63	Santa Cruz Biotechnology (Cat no SC-8431)	Mouse	Monoclonal	1 in 500	200µg/ml
Anti-Smooth Muscle Actin	Sigma Aldrich (Cat no A2547)	Mouse	Monoclonal	1 in 5000	N/A
Anti-DIG-AP	Roche (Cat no 11787126)	Sheep	Polyclonal	1 in 2000	750U/ml
Anti-EphrinB1	R&D Systems (Cat no AF473)	Goat	Polyclonal	1 in 250	100µg/ml
Anti-EphrinB2	R&D Systems (Cat no AF496)	Goat	Polyclonal	1 in 200	100µg/ml
Anti-EphB2	R&D Systems (Cat no AF467)	Goat	Polyclonal	1 in 100	100µg/ml
Anti-EphB3	R&D Systems (Cat no AF432)	Goat	Polyclonal	1 in 50	100µg/ml

2.1.8 Kits

Qiaquick PCR Purification Kit (Cat no 28174), QIAprep Spin Miniprep Kit (Cat no 27104), and the RNeasy Mini Kit (Cat no 74106) were all purchased from Qiagen.

TOPO TA Cloning Kit for Sequencing Kit with pCR4-TOPO Vector and TOP10 Chemically Competent *E.coli* (Cat no 45-0071) were purchased from Invitrogen.

TURBO DNA Free DNAse Kit (Cat AM1907) was purchased from Ambion.

PowerSYBR Green PCR Mastermix (Cat no. 4367659) was purchased from Applied Biosystems.

2.1.9 Standard Solution (A-Z)

Bouin's Fixative	75 ml saturated picric acid 25 ml 40 % (v/v) Formaldehyde 5 ml glacial acetic acid
Paraformaldehyde fixative	4 % (w/v) paraformaldehyde in PBS
PBS	Phosphate buffered saline tablets supplied by Sigma®
SSC (20 X)	3 M NaCl, 0.3 M sodium citrate pH 7.0
TAE (50 X)	2 M Tris acetate, 50 mM EDTA ph 8.0
TBST	0.15 M sodium chloride

	0.1 % Polyoxyethylenesorbitan Monolaurate Tween20, in Tris pH 7.4

2.1.9 WISH Solutions (A-Z)

Antibody Solution	Anti-DIG (1/2000) in TBST with 1 % sheep serum
Blocking Solution	10 % sheep serum in TBST
Hybridisation Solution	50 % Formamide, 5XSSC, 2 % blocking powder, 0.1 % Triton X100, 0.5 % CHAPS, 5 mM EDTA, 50 μg/ml, heparin, 1 mg/ml Yeast RNA
Hydrogen Peroxide	6 % Hydrogen Peroxide in PBST
NTMT	100 mM Tris-HCl pH9, 100 mM NaCl, 50 mM $MgCl_2$, 0.1 % Tween20
Post – fixation Solution	4 % Paraformaldehyde, 0.2 % Glutaraldehyde, 0.1 % Triton X100, in PBST
Proteinase K	20 μg/ml in PBST
Solution I	50 % Formamide, 5X SSC, 0.1 % Triton X100, 0.5 % Tween20
Staining Solution	2 mM Levamisole, NBT, BCIP in 10 ml NTMT

2.2 Methods

2.2.1 Whole mount RNA In-situ Hybridisations

All whole mount in-situ hybridisations (WISH) were performed using the Invatis AG Insitu Pro VS robot. A universal protocol was devised for all WISHs conducted using a hybridisation temperature of 65°C. Probes were heat denatured at 65 °C for 5 minutes before being added to 750 µl Pre-hybridisation mix per well. Dehydrated tissues were placed in 700 µl of 100 % methanol at the start of each WISH experiment. After the WISH experiment (as outlined in Table 2.2), each tissue sample was transferred from TBST solution to a fresh 24 well plate and incubated at 37°C for 2-8 hours in 1 ml NTMT staining solution prior to being photographed. Some tissue samples required exposure to the NTMT staining solution overnight at 4°C.

Tissue collection and fixation

All tissue was collected from Wistar *Rattus norveguis* rats at d0. The urogenital tract was micro-dissected from both males and females. Tissues were fixed in 4 % paraformaldehyde and stored in methanol. Tissues used in the experiments described in this thesis include, from males: ventral prostate, anterior prostate, and entire male urogenital tracts and, from females: entire female urogenitial tracts. The urogenital tracts micro-dissected did not contain kidneys.

Whole mount RNA In-situ Hybridisation Treatments

Stage	Treatment	Number of Washes	Length of Wash (mins)	Temp (°C)
1	50% Methanol	2	40	Room Temp
2	PBST	3	40	Room Temp
3	6% H2O2	1	40	Room Temp
4	PBST	2	40	Room Temp
5	Proteinase K	1	60	Room Temp
6	PBST	1	40	Room Temp
7	Postfix Solution	1	20	Room Temp
8	PBST	1	20	Room Temp
9	PBST	2	40	Room Temp
10	Hyb Solution	1	40	Room Temp
11	Hyb Solution	1	40	65
12	Hyb Solution	1	120	65
13	RNA Probe	1	16 hours	65
14	Solution I	2	40	65
15	2X SSC/Solution I	2	40	65
16	2X SSC	3	40	65
17	0.2X SSC	3	40	65
18	TBST	2	40	Room Temp
19	Blocking Solution	1	120	18
20	DIG Antibody	1	6 hours	18
21	TBST	1	40	Room Temp
22	TBST	3	60	Room Temp
23	TBST	3	90	Room Temp
24	TBST	2	120	Room Temp

2.2.2. RNA probe production

RNA Extraction and cDNA synthesis

All RNA extracted was obtained using RNeasy Mini Kits (Qiagen) and quantified using the Nanodrop Spectrophotometer ND-1000. cDNA synthesis was performed using 500-750 ng RNA per reaction. cDNA synthesis was conducted in a final volume of 20 μl. RNA (made up to 10 μl with pure water) was incubated at 70 °C for 5 minutes with 1 μl of 10 mM dNTPs and 1 μl Oligo DT (25 pmol/μl). A mixture of 4 μl 5X First Strand Buffer (Invitrogen), 2 μl 0.1 M DTT (Invitrogen) and 1 μl RNAsin (Promega) was added to each reaction. Reactions were incubated at 42 °C for 50 minutes. After two minutes, 1 μl Superscript II RNAse H- reverse transcriptase (Invitrogen) was added to each reaction. cDNA was diluted using RNAse free water to a final volume of 100 μl per reaction.

Polymerase Chain Reactions (PCR)

All PCRs were performed using the Peltier Thermal Cycler PTC 200 (MJ Research). All PCRs were conducted for 35 cycles. All PCR primers for SAGE derived molecules were designed from appropriate rat or mouse NCBI GenBank sequence using Vector NTI (Invitrogen) and ordered from MWG Biotech. All primer sequences were checked using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to ensure no homology existed outside the intended sequence for amplification. PCRs were performed using Platinum Taq polymerase (Invitrogen), 10 X buffer (200 mM Tris-HCl (pH 8.4), and 500 mM KCl), 10 mM dNTPs, 50 mM Mg2+ (Invitrogen). PCR product purification was performed using the QIAquick PCR purification kit (Qiagen). The standard PCR thermal cycling conditions were as described below. The melting temperature varied between 58-62°C for all the primer pairs.

95 °C – 2 minutes

95 °C – 30 seconds

58-62 °C – 30 seconds | x35 cycles

72 °C – 45 seconds

72 °C – 10 minutes

4°C – ∞

For PCR product visualisation, 5 µl of each reaction was ran on a on 2 % agarose gels using 1X TAE Buffer. Gels were visualised using Syngene Bio Imaging GeneFlash UV transilluminator.

Table 2.3. Primer information for the Statistically Selected Transcripts. The forward primer for each gene is in the first row and the reverse primer in the second row.

Gene	Unigene	Primers	GenBank File	PCR Product Size (bp)	Tm (oC)
RasD	Rn.11189	TCACAACGTCCAGGCACTGT	NM_053338	456	59
		GGGTTCAGGCTGCTCGGTAT			
Nell2	Rn.11567	TGAGTGCGACCCAAGGCTGA	NM_031070	603	N/A
		TCCGTAAGGTGATCCCAGGA			
Cystatin	Rn.9609	CTTGGCCTGCTCGCATTCTG	NM_133566	489	60
		TCATGACCCTGAGGTGCCTC			
Glutamate Rich Protein	Rn.33523	GGATGTCAACGTCATCAGTT	NM_001012067	592	58
		GCTCAGTCCAAATCATTAGA			
ANP32A	Rn.10123	GTGACCAACCTGAATGCCTACC	NM_01293	460	61
		TTTCGGGGGGCAGGAATAGG			
Ubiquitin Interacting Protein	Rn.103261	TCTCAGTCTCTTGGAGCAGT	NM_001013884	423	60
		ACATAAAAATCAGGGTGCTG			
PDZ Ring Finger 3	Rn.3111	TGAAGAGCGGAAGCTCATCC	XM_232226	577	59
		GAAGGCCATCCCATCATTCC			
TRAF Interacting Protein	Rn.8891	GATCACGAGCCTAAGAAAGA	XM_345981.2	503	61

		CTGTAGGTTGGATGAATTTT			
Vaccina Related Kinase 3	Rn.6570	GATAACTAAGCAGAAGCAGA	NM_001005561	482	61
		ACATCACAGGAAGGTCCTAT			
EphB3	Rn.131133	AGGATGTCATCAATGCCGTA	NM_001105868	547	62
		AGGCCAGGTATCCAAAAGTC			

Table 2.4. Primer information for the Non-Statistically Selected Transcripts. The forward primer for each gene is in the first row and the reverse primer in the second row.

Gene	Unigene	Primers	GenBank File	PCR Product Size (bp)	Tm (oC)
SC43M1	Rn.35465	TGGTTACATCATGGACTGGC	XM_230050.3	682	61
		GCCCCTGGCTCTATGCATAC			
CSC4	Rn.55346	GCAGGGATGCCTGGAGTAGA	XM_342506.2	492	N/A
		GACGTCTTCCTCTCCACCAT			
NMB	Rn.13778	CTCTGGGAGACGATGCAAGC	NM_133298.1	593	60
		GCATCCTCTCCCAGTTAACC			
Nuclear Enzyme	Rn.6997	GTACTTCTGCGGGAGCATCC	NM_133525	443	58
		GAGGAAGCCGTCTTCTGAGG			
Ras-related GTP Binding A	Rn.2816	ATGGTGGTCATGTCAGATCC	NM_053973.2	371	61
		CCCATACACACCACCTACTT			
Cyclin G Associated Kinase	Rn.9560	CCTTCCTGCGCTGCTGCTTT	XM_215403	613	N/A
		TCCGGACTGCACTCGGGCTG			
MARCKS	Rn.11012	GAGCCTCTGAACAACTAAGG	NM_031030	652	60
		GATGAATGCAGGGATGATTG			
CRIP2	Rn.94754	GCAGGAAAAATTTAGACAGC	NM_177425.3	565	60
		TGTGCTACACAGTGCTGAGT			

Acyl-CoA Thioesterase	Rn.11326	CAACTGGAAGAGCGAGTTCT	NM_031315	504	N/A
		CCTTACAGCACGGAGATTGA			
ETL	Rn.8731	CTGGTCTGCTCTATCATCGC	NM_022294	514	60
		ACATCCCTTGGAAAGCGTTG			
Transcribed Protein	Rn.25416	GGCACCATTCATTGAGTTTT	NM_198771	487	61
		TCAGTGATTGGCTCAATGAG			
Smu1 Suppressor	Rn.54978	CAAGATGGGCATTACATTAT	Nm_057195	592	62
		AACCCGTAGCTACTGACTTA			
Kruppel-Like Factor 5	Rn.8954	TGCCACTCTGCCAGTTAATT	NM_022690	509	62
		CAAAATGACCAAACACTTTG			
Hypotheical Protein	Rn.94991	CATGGCCATGTCTTGCATCG	XM_215812	504	58
		CCCACGACCACGAGGTGTAA			
Bromodomain Containing 3	Rn.16796	CCAATGGACATGGGGACTATC	XM_342396	534	N/A
		AGTGATGGCGGACGTTGTGG			
Bromodomain Containing 4	Rn.12110	AAGCATCCGACCACCCCATC	XM_343175.2	537	N/A
		CTGCCGCCCCTAACACTATG			
Tetraspanin 4	Rn.2146	AGATTCCGTGAAGTGTTTGC	NM_001015026	509	61
		TTTCTGTCATTTCCAACCAA			
P3K Protein	Rn.44193	AGAGACTTACGATGGATATTTC	BC099154	571	N/A
		CATCATGGAGAAAAGGTTGA			
Helicase DNA Binding Protein 4	Rn.63247	ATTGATGGTGGAATCACTGG	XM_232354	547	N/A
		TCAAATATTCATTCATGCCC			
Initiation Factor 1A	Rn.3361	TGATCAAAATGCTGGGAAAC	NM_001008773.2	534	62
		CCAGAAGCACAAAGTGTTAA			
Evectin 2	Rn.1410	TATGCTACACCGACCCCTGA	XM_217372.3	551	61
		CTGAACGGAAAATGCCCTTT			
F Box Protein 2	Rn.103250	TGAGACATGGCTTGATAACA	XM_231162	526	61
		AACACACTGCCCTGTGCTTA			
Myc Binding Protein 2	Rn.9802	CCGTTTAAATGTCTTCTGAT	XM_214245.3	532	N/A
		ATTCTCCTCTTCCTCCAAAC			

Laminin 2	Rn.21475	TAGAACAGGAAGCTGATCGG	XM_219866	541	N/A
		CCAACAAACAGCATTGCATT			
Adrenal Gland Protein	Rn.56498	AGCACGCCTTCAATGAGCAC	BC099150	599	62
		TTGGAACTTGCCCACATAGA			
SCC3	Rn.101480	CAGCTCTTGTGCAGCAGGAAG	XM_222279.3	523	60
		AGGGCCTGCTGGGCTGACTT			
Rbms1	Rn.98522	CACACCATGTCACTACAACC	NM_001012184.1	455	60
		GGGAACAGCTACTAGATGAA			
PIK3ca	Rn.44193	ATTTGGCTATAAACGGGAAC	BC099154	523	61
		TTCAATTATAGAGCACGTTG			

Table 2.5. Primer information for the Intuitive Transcripts. The forward primer for each gene is in the first row and the reverse primer in the second row.

Gene	Unigene	Primers	GenBank File	PCR Product Size (bp)	Tm (oC)
SPARC	Rn.98989	CCCGAGACTTTGAGAAGAAC	NM_012656.1	518	60
		AGGCTGTGGATAGGCTATGG			
DECORIN	Rn.106103	CGAAGACACATCTGAAGGTG	NM_024129.1	523	62
		CAGTCAACTGCATCTGGATG			
TSUKUSHI	Mm.25317	CCAAGCTCAAGTGGGCAG	NM_001009965.2	503	62
		GCATCGAAGTCCCTTTGC			
SPRY1	Rn.27787	CAAGCCGTCATGACTTCTGG	NM_001106427.1	535	62
		GTGAATCCAGAGCTGTGTGC			
NELL2	Rn.11567	GGAAACCGTGTACAACAGCG	NM_031070	514	60
		GGTTTCCTCAGTGATGTTCC			
SEMA6D	Rn.8257	TTCTGCCACAGTGGCTGATT	XM_230583	548	60
		GCCTTGGTTTTGGTACTTTG			
SORCS2	Rn.41133	GAGGAGCTACTAGTGACTGTG	NM_001107225.2	564	59
		GTGTGTCGTTCCAGCTGTAC			
CMTM3	Rn.15322	AGGGGAAATAGAGAAGACAG	XM_226200	538	59
		AGTCAATGGCAAACACAATT			

ILGF1	Rn.6282	GTACCAAAATGAGCGCACCT	NM_178866	547	60
		CCATCTCTGAAATGGTATTGAACTC			
ILGF2	Rn.118682	AGTCAGTTTGGCCAGATAAG	X16703	531	N/A
		TACTGGCTCTCGCTGGTATT			
LTBP3	Rn.202129	GCGGGGCGGTATGCGAGTGT	NM_008520	522	N/A

Table 2.6 Primer information EphB PCR Analysis. The forward primer for each gene is in the first row and the reverse primer in the second row.

Gene	Primer Sequence	GenBank File	PCR Product Size (bp)
EphB1 F	AACACCCTGGACAAGATGAT	NM_001104528.1	561
EphB1 R	GTCTTTATCGTCAATCATTACG		
EphB2 F	CCCAAGTTCGGCCAGATCGT	NM_001127319	527
EphB2 R	GGTGACGGCTTGACTGGAGT		
EphB3 F	AGGATGTCATCAATGCCGTA	NM_001105868.1	547
EphB3 R	AGGCCAGGTATCCAAAAGTC		
EphB4 F	ATGCTGGACTGTTGGCAGAA	NM_010144.5	550
EphB4 R	AATCCTGTGTCTCCGACCCC		
EphB6 F	AGACACTTGGCAGAAGGACC	NM_001107857.1	510
EphB6 F	GCTGGGTTTAATGTGGCAGG		
EphrinB1 F	CAGCCGGCCAAGCAAAGAGT	NM_017089.2	568
EphrinB1 R	GGGGAGAAAAGAGAGGCCAA		
EphrinB2 F	TATATCTACATCAAATGGGTC	NM_010111	481
EphrinB2 R	AGTCCTTAGCGGTATGATAA		
EphrinB3 F	GCCTAACCAGAGGCATGAAGGTGC	NM_001100980.2	663
EphrinB3 R	GCAAAGGGGGCCAAAGTCAT		
GADPH F	TTAGCACCCCTGGCCAAGG	NM_002046	541
GADPH R	CTTACTCCTTGGAGGCCATG		

Candidate specific cDNA sequence isolation

The purified PCR products used for RNA probe production were cloned into the pCR4-TOPO cloning vector (Invitrogen) and transformed into chemically competent TOP10 *E.coli* cells (Invitrogen). Ligation and transformation were conducted according to the manufacturers protocols. Cells were grown on LB/Ampicillin plates overnight at 37 °C. One colony for each PCR product was picked and used to inoculate a 10 ml LB/ampicillin broth culture incubated shaking overnight at 37 °C. Each pCR4-TOPO plasmid with insert was extracted using QIAprep Spin Miniprep Kit (Qiagen). Plasmid preparations were quantified using the Nanodrop ND-1000 UV/Vis spectrophotometer.

Restriction Enzyme (RE) digestions

pCR4-TOPO contains restriction enzyme sites for *Not I* and *Spe I* on the 5' and 3' sides, respectively, of the multiple cloning site (MCS). pCR4-TOPO also has *Eco R*1 restriction sites on both sides of the MCS. *Not I* (Promega) and *Spe I* (Roche) were used to linearise each plasmid preparation. The *Not I* digest contained the T3 RNA polymerase promoter and the *Spe I* digest contains the T7 RNA polymerase promoter. All digests were purified using the QIAquick PCR purification kit (Qiagen). Each RE reaction had varying final volumes. Between 1-2 μg DNA was used for each digest. Digests were incubated at 37 °C for 2-3 hours. An *Eco RI* digest was also to confirm that the cloning had succeeded. For digest visualisation, 5 μl of each *Not I/Spe I* reaction and all of each *Eco RI* and was run on a 2 % agarose gel using 1X TAE Buffer and stained with ethidium bromide. Gels were visualised using Syngene Bio Imaging GeneFlash UV transilluminator (Figure 2.1).

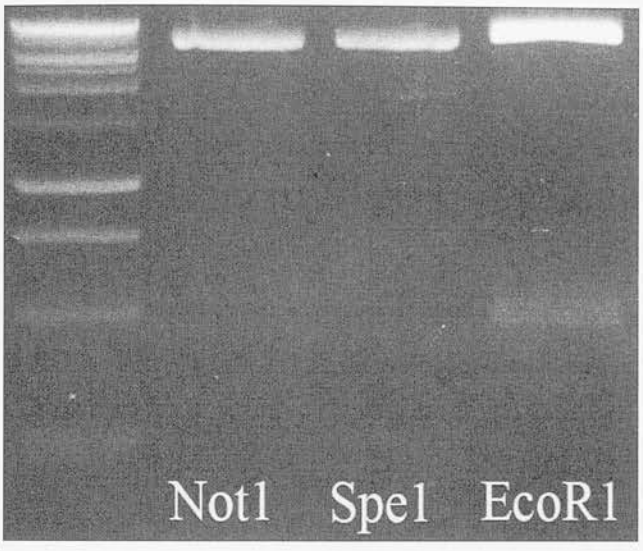

Figure 2.1 Restriction Enzyme Digest Gel. The Not1 and Spe1 digests show a linearised plasmid whereas EcoR1 has cut the insert out to give am insert (approximately 500bp).

DIG labelling *In Vitro* transcription

Each transcription reaction volume was 20 µl. Between 400-600 ng DNA was used per transcription. T3 RNA Polymerase (Roche) was used for *Not I* Digests and T7 RNA Polymerase (Roche) was used for *Spe I* digest. Each transcription used 10X Roche transcription buffer, 2 µl DIG/NTP labelling mix (Roche) and 0.5 µl RNAsin (Promega). Reactions were incubated at 37 °C for 2 hours and then incubated with 2 µl DNAse 1 (Promega) for a further 30 minutes. RNA probes were stored at -20 °C until use. For DIG labelled RNA probe visualisation, 2 µl of each transcription reaction was run on a 2% agarose gel using 1X TAE Buffer and stained with ethidium bromide. Gels were visualised using Syngene Bio Imaging GeneFlash UV transilluminator.

2.2.3. *In Vitro* Organ Culture

VPs and APs were micro-dissected from d0 male Wistar rats and grown in serum-free organ culture media (DMEM/NUT.MIX F-12 supplemented with transferrin (20 μg ml^{-1}), insulin (20 μg ml^{-1}), and 1 X penicillin/streptomycin). VPs and APs were positioned in a drop of media on Millicell® CM filters that were floated on 1 ml of culture media in 4 Well NUNCLON™ plates. Organs were cultured at 37 ^{0}C in 5 % CO_2 for six days. Media (+/- treatments) was replaced every two days. Proliferation of epithelial and stromal cells in VPs was examined by incorporation of BrdU (0.1-0.2mg/ml) on day six of culture. The BrdU incorporation involved incubation for 2 hours at 37 ^{0}C. VPs were imaged by light microscopy before fixation. To study the effect of EphB2 and EphB3 ligand trap on VP and AP area and the number of epithelial buds, VP and AP organs were cultured in the absence or presence of testosterone (10^{-8} M) and/or either EphB2-Fc (4 μg/ml), EphB3-Fc (4 μg/ml) or both. To study the effect of EphrinB1 and EphrinB2 ligand addition on VP and AP area and the number of epithelial buds, organ rudiments were cultured in the absence or presence of testosterone (10^{-8} M) and/or either EphrinB1-Fc (4 μg/ml) EphrinB2-Fc (4 μg/ml) or both. A control Fc (4 μg/ml) was also included in all experiments.

2.2.4. Quantitative RT-PCR

All RNA extractions and cDNA synthesis reactions were performed as described above. 400ng total RNA was used for each cDNA synthesis reaction. The 20 μl cDNA reaction volume was diluted to a final volume of 100 μl. 5 μl of this 100 μl was made up to a final volume of 50 μl as a working stock. The quantitative PCR system used for all reactions was PowerSYBR Green (Applied Biosystems) and results were normalised to

TATA Binding Protein (TBP). Each final primer concentration for each quantitative PCR reaction was 6.25pmol/µl (1:4 dilution of 25pmol/µl working stocks). All PCR products were approximately 100bp in length. Table 2.7 contains all the primer sequences used in all the quantitative PCR reactions. Each quantitative PCR reaction had a total volume of 10µl and consisted of the following:

2X PowerSYBR Mastermix -5µl
Forward Primer - 1µl
Reverse Primer - 1µl
Water - 1µl
cDNA - 2µl

A standard curve was determined for each gene analysed by quantitative PCR using the following dilutions of VP d0 – 1:5, 1:25, 1:100, 1:500 and 1:1000. The thermal cycling conditions used for each reaction:

50 °C – 2 minutes
95 °C – 10 minutes
Then 40 cycles of the next two steps:
95 °C – 15 seconds
60 °C – 60 seconds
Then dissociation curve:
95 °C – 15 seconds
60 °C – 15 seconds
95 °C – 15 seconds

Table 2.7 Primers used for quantitative PCR experiments.

Primer	Primer Sequence
EphB3 F	GTGCCTCCCAGAGACTGACT
EphB3 R	CAAGCATGTGCACAACACAC
EphB2 F	CCGGCTTCACCTCTTTTGA
EphB2 R	CCGCATCACCTGGATACTGT
EphrinB1 F	CCCCCACTATGAGAAGGTGA
EphrinB1 R	GGGCCCTCAGACCTTGTAGT
EphrinB2 F	CCAAGAATTCAGCCCTAACC
EphrinB2 R	GTTATCCAGGCCCTCCAAAG
TBP F	CTGGAAGGCCTTGTGTTGAC
TBP R	GGAGAACAATTCTGGGTTTGA

2.2.5. Immunohistochemistry (IHC)

Paraffin Embedding of Tissue

Micro-dissected tissue or cultured VPs were fixed in 4 % PFA, for WISH, or Bouins fixative, for IHC, for 2 hr on ice. Tissue was stored in 70 % ethanol until processed. Dehydration and further processing was performed on a Leica TP 1050. Paraffin embedding was performed on the Tissue Embedding Center (Leica EG1160) and 5 μm sections cut using a microtome (Leica RM2135).

Hematoxylin and Eosin Stain

Slides were washed twice in Xylene for 5 minutes to for de-waxing purposes. Slides were exposed to 20 second washes of Absolute Alcohol, 95 % Ethanol, and 70 % Ethanol. Slides were then washed in water. Slides were then exposed to hematoxylin for 5 minutes. Slides were then washed in water. Slides were then exposed to Acid Alcohol

for 20 seconds, Scott's Tap water for 30 seconds, and Eosin for 20 seconds with a water wash in-between each step. Slides where then exposed to treatments for 20 seconds each. These treatments were 70 % Ethanol, 80 % Ethanol, 95 % Ethanol, and Absolute Alcohol. Slides were then washed twice in Xylene for 5 minutes and mounted.

3,3'-Diaminobenzidine (DAB) based Immunohistochemistry

Slides were dewaxed in xylene for 10 minutes and rehydrated through an ethanol series. Slides were pressure cooked for 5 minutes in 0.1 M citrate buffer, cooled for 20 minutes and washed twice in TBST on a platform shaker for 5 minutes. Slides were washed in 6 % Hydrogen Peroxide in methanol solution for 30 minutes on a platform shaker to eliminate any endogenous peroxidase activity. Slides were washed twice in TBST on a platform shaker. Slides were blocked in 20 % normal serum (serum of the host the secondary antibody was raised in) in TBST at room temperature for 60 minutes, followed by an overnight incubation at 4°C with the appropriate primary antibodies diluted in 20 % normal serum in TBST. For a list of all the primary antibodies used, see Table 2.1. Slides were washed three times for 5 minutes in TBST followed by incubation for 1 hr at room temperature in the required IgG-biotinylated secondary antibodies diluted in 20 % normal serum in TBST. For a list of all the secondary antibodies used, see Table 2.8. Slides were washed twice for 5 minutes in TBST and twice for 5 minutes in PBS. Slides were then incubated for 60 minutes with Horseradish Peroxidase Strepavidin (Vector Laboratories, Cat number SA5004) at 1 in 1000 dilution in 20 % normal goat serum. Slides were washed twice in PBST on a platform shaker and then stained using the DAB Substrate Chromogen System (Dako, Cat number K3468). Slides were observed under light microscopy until a brown stain developed. Slides were washed in water and counterstained with hematoxylin and rehydrated through an ethanol series. Slides were visualized using an Olympus Provis AX70 microscope and pictures taken using a Canon EOS 30D camera.

Table 2.8 Secondary antibodies used and the primary antibodies utilised with DAB based immunohistochemistry.

Primary	Secondary	Source and Dilution
Anti-p63 Anti-SMA	Rabbit Anti-Mouse Biotinylated	Dako (Cat E0464) 1 in 500
Anti-EphrinB1 Anti-EphrinB2 Anti-EphB2 Anti-EphB3	Rabbit Anti-Goat Biotinylated	Vector Laboratories (Cat BA5000) 1 in 500

Fluorescent Immunohistochemistry

Sections were dewaxed (in xylene) for ten minutes and rehydrated through an ethanol series. Sections were pressure cooked for 5 minutes in citrate buffer, cooled for 20 minutes and washed twice in TBST on a platform shaker. Sections were blocked in 20 % normal serum (serum of the host the secondary antibody was raised in) in TBST at room temperature for 45 minutes, followed by an overnight incubation with the appropriate primary antibodies diluted in 20 % normal serum in TBST. For a list of all the primary antibodies use, see Table 2.1. Slides were washed three times for 5 minutes in TBST followed by incubation for 1 hr at room temperature in Rabbit anti-Sheep biotinylated secondary antibody (Vector Laboratories, Cat number BA6000) diluted in 20 % normal serum in TBST. Slides were washed twice for 5 minutes in TBST and twice for 5 minutes in PBS. Sections were then incubated for 1 hours with either Strepavidin Alexaflour 488 at 1 in 200 dilution (Molecular Probes) or Goat anti-mouse CY5 at 1 in 60 dilution (Sigma Aldrich) diluted in 5 % normal goat serum. Slides were kept in opaque box after this step, as the fluorescent detectors are light sensitive. Slides were washed in PBST overnight at 4°C. Nuclei were counterstained using 10 $\mu g\ ml^{-1}$ propidium iodide in PBS for 45 minutes, followed by three washes in PBS for 5 minutes

each. Sections were visualised and imaged using confocal microscopy (Zeiss LSM 500). The Fluorescent IHC protocol and the antibodies used are summarised in Table 2.9.

Table 2.9 Summary of antibodies used in Fluorescent Immunohistochemistry

IHC	Primary	Secondary	Detector
Pan-cytokeratin and BrdU	Anti-cytokeratin (pan) 1:200	Rabbit anti-sheep biotinylated 1:500	Goat anti-mouse CY5 1:60
	Anti-BrdU 1:300		Streptoavidin alexafluor 488 1:200

3 Identification of Mesenchymally Expressed Transcripts using Whole mount In-Situ Hybridisation (WISH)

3.1 Introduction

The objective of this study was to identify genes expressed in the stroma of the rat prostate and to elucidate their function. A transcript profiling study using Serial Analysis of Gene Profiling (SAGE) had been previously conducted with the aim of identifying molecules enriched in the ventral mesenchymal pad (VMP), an area of condensed inductive mesenchyme (Vanpoucke et al., 2007), known to express key morphogens (Thomson and Cunha., 1999) and possess prostate inductive activity (Timms et al., 1995). The expression profile of the VMP was compared with the VSU which encompassed the VMP, the SM layer and the urethral epithelium. This study revealed 219 mRNAs upregulated in the VMP compared to the VSU. The SAGE analysis and its outcome are summarised in Figure 3.1. It is likely that some of these 219 transcripts are involved either in paracrine signalling between the VMP and urethral epithelium and smooth muscle or juxtacrine signalling within the VMP itself. Both paracrine and juxtacrine signalling are known to have key roles in rat prostate development.

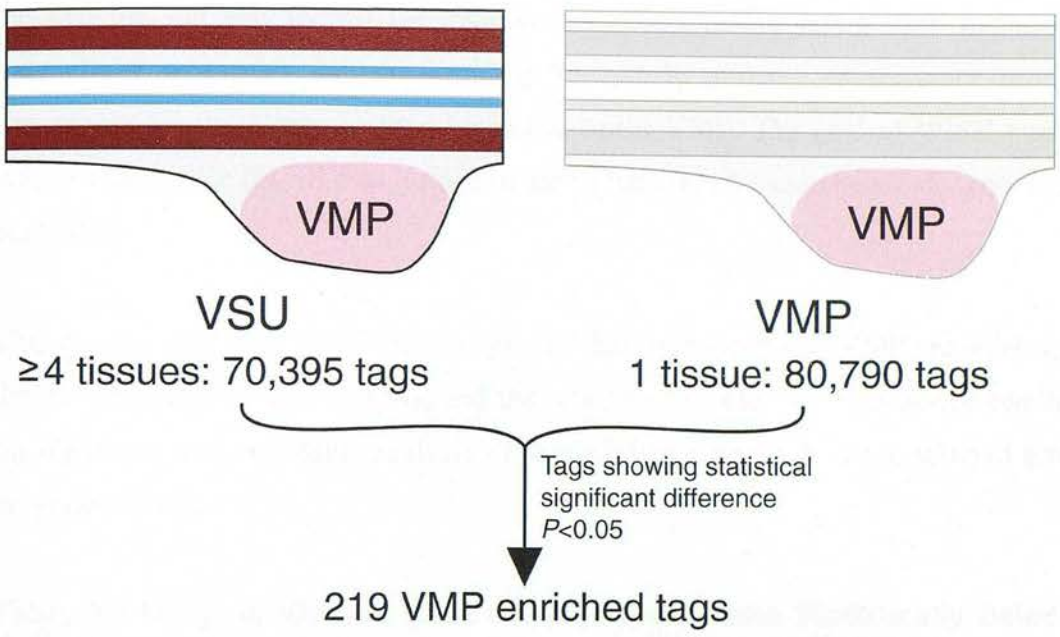

Figure 3.1 Summary of the SAGE analysis performed on the VSU and the VMP (derived from Vanpoucke et al., 2007).

The SAGE analysis identified transcripts likely to be expressed in the VMP. Three groups of mRNAs were identified from the study for follow up. These three groups were to be examined using whole mount in-situ hybridisation (WISH) to confirm the VMP specific expression of the transcripts. The difference between two of the three groups was their statistical significance. The minimum threshold for statistical significance between the VMP and VSU mRNA tag counts was a ratio of 5 (VMP) to 0 (VSU). The 5:0 group was a subset of the 219 list (53 transcripts in the 5:0 group). It was determined that all the transcripts with an expression ratio of 5:0 would be included in the WISH analysis and labelled the statistically selected group. As a control to the statistically selected group, all the transcripts that showed a ratio of 4:0 were included in the WISH analysis. As the 4:0 group did not show a statistically significant difference, this group of transcripts could be considered as a randomly selected group. The rationale behind the selection of the two groups was that the WISH analysis of the two groups

would determine the efficiency of the SAGE analysis in identifying VMP specific transcripts. The third group to be included in the WISH analysis consisted of genes coding for either membrane or secretory proteins. This third group was also a subset of the 219 list and was termed the intuitive list. The intuitive group were included as intercellular molecules that are typically membrane proteins or secretory proteins. Transcripts for these may be found enriched in the VMP. The goal of WISH analysis was to validate the SAGE data, to determine which mRNAs were genuinely enriched in the VMP.

This chapter discusses the WISH analysis of the three groups of VMP candidates, the degree of success of this analysis, and the rationale for selecting a particular candidate for functional and expression analysis. The candidate genes in the three selected groups are shown below.

Table 3.1 Unigene IDs and gene descriptions for the Statistically Selected Transcripts. These all showed a tag frequency of 5:0 in VMP: VSU list.

Unigene	Description
56603	Amine oxidase, copper containing 3
1868	Beta-2 microglobulin
8046	Casein kinase 1, delta
9609	Cystatin N
2342	Mannosyl Glycoprotein beta-1,2-N-acetylglucosaminyltransferase
11567	Nel-like 2 homolog
99017	Pr2 protein
53915	PV-1
11189	Ras-related associated with diabetes
9560	Rat mRNA
16016	Eps protein (MGEPS) mRNA
33523	A301 protein (LOC308592)
16844	Amyotrophic lateral sclerosis 2 chromosome region candidate 9;
36521	FGFR1 oncogene partner (LOC365103)
52627	Hypothetical protein (LOC366980)
61277	Hypothetical protein D930024B17 (LOC303552)
2918	MUM2 protein (LOC287427)
100904	Pmcf-pending protein (LOC297342)
103261	Retinoid x receptor interacting protein (LOC290997)
103390	RIKEN cDNA 0610008A10 (LOC300802)

8133	RIKEN cDNA 1110038M16 (LOC313529)
98420	RIKEN cDNA 1700027N10 (LOC316238)
3111	Semaphorin cytoplasmic domain-associated protein 3A
93304	Similar to TRAF-binding protein (LOC293571), mRNA
6570	Similar to vaccinia related kinase 3 (LOC361565), mRNA
117190	Transcribed sequence
116313	Hypothetical protein DKFZp564D0372
118492	RS15_HUMAN 40S ribosomal protein S15 (RIG protein)
6532	DNAJ domain-containing
27227	Apoptosis regulator Bcl-2
16544	Patched homolog 1
29900	Cbx1
14510	Myelin protein zero-like protein 1
8458	Paired mesoderm homeobox protein 1 (PRX-1)
91975	Phosphoinositide-3-kinase, class 2, beta polypeptide
17732	Sox7_predicted, 'SRY-box containing gene 7 (predicted)
23241	GLI-Kruppel family member HKR2 2
1078	Spindlin-1
28360	ADAM-TS12
41830	zinc finger protein 236
121231	cyclin M3
103231	Scube1
66545	Pleiomorphic adenoma gene 1 (PLAG1)
62699	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1
24283	Amyloid beta (A4) precursor protein-binding, family B, member 2 (Apbb2)
8202	Ubiquitin-conjugating enzyme UBC7
3455	Poly (A) polymerase alpha
131133	Eph receptor B3
2528	Cytochrome c oxidase subunit 1
10123	Acidic leucine-rich nuclear phosphoprotein 32 family member A
146652	Slit-2
103639	GPR177
377046	Activating transcription factor 6 alpha

Table 3.2 Unigene IDs and gene descriptions for the Non-Statistically Selected Transcripts. These are transcripts that showed no statistical difference between the VMP and VSU libraries. Genes with (PS) had only partial similarity to a known putative gene.

Unigene	Description
5041	ATPase, Na+/K+ transporting, beta 3 polypeptide
3733	CD164
33804	CD74
10204	Cell division cycle 2 homolog like 1
3974	CDC31
2819	Coronin, actin-binding protein, 1B
93	CTD-binding SR-like rA1
11012	Cyclin G-associated kinase
94754	Cysteine rich protein 2
11326	Cytosolic acyl-CoA thioesterase 1
8731	ETL protein (EGF_TM7_Latrophilin Protein)
25416	FAM3C-like protein
15845	Fc receptor, IgG, alpha chain transporter
13778	Glycoprotein (transmembrane) nmb
54978	Homolog of C. elegans smu-1
8954	Kruppel-like factor 5 (intestinal)
44193	Phosphatidylinositol 3-kinase
1653	Pleiotrophin
6997	Putative c-Myc-responsive
2816	Ras-related GTP-binding protein ragA
94991	Hypothetical LOC302941 (PS)
13437	53BP1 protein (PS)
16796	Brd3 protein (PS)
12110	BRD4 short variant (LOC362844) (PS)
3424	cDNA sequence BC013529 (LOC361448) (PS)
7522	Cgi67 serine protease precursor (LOC309399) (PS)
63247	Chromodomain helicase DNA binding protein 4 (PS)
11938	DNA-directed RNA polymerase II largest subunit (PS)
3361	EIF-1A (LOC317163) (PS)
2146	EST AI426782 (PS)
1410	Evectin-2 (PS)
103250	F-box protein FWD2 (PS)
55346	H63 breast cancer expressed gene isoform a (PS)
13538	Highwire; PAM; rpm 1 (PS)
58352	Hypothetical protein (LOC305628) (PS)
17560	Hypothetical protein (LOC361091) (PS)
4001	Hypothetical protein 5832424M12 (LOC309129) (PS)
34037	Hypothetical protein FLJ20522 (LOC288041) (PS)
41869	Hypothetical protein FLJ32734 (LOC304509) (PS)
1698	Integral membrane protein CII-3 (LOC289217) (PS)
11970	KIAA0404 (LOC309180) (PS)
56498	Adrenal gland protein AD-005 (LOC291841) (PS)

35504	KIAA1417 protein (LOC315858) (PS)
21475	Laminin-2 alpha2 chain precursor (LOC309368) (PS)
101349	Low-density lipoprotein receptor-related protein 10 (PS)
57349	Microtubule-actin crosslinking factor (LOC298511) (PS)
67	Mitochondrial ribosomal protein S14 (LOC289143) (PS)
35465	Prostate cancer overexpressed gene 1 (LOC311168) (PS)
76647	Protein kinase NYD-SP15 (LOC361052)
22892	WDC146 (LOC307524)
63701	Rab20 (LOC364641)
110990	RIKEN cDNA 1110021N07 (LOC362912)
39346	RIKEN cDNA 1600029D21 (LOC363060)
49051	RIKEN cDNA 2310042P20 (LOC300189
7240	RIKEN cDNA 2510005D08 (LOC290279)
102152	RIKEN cDNA 6720485C15 (LOC360639)
98522	RNA binding motif, single stranded interacting protein 1
20004	SWI/SNF-related matrix-associated protein
101480	SART3
54356	Lactose Operon Repressor Bound
21367	T46908 hypothetical protein DKFZp761G2423.1
21890	2115356A p15 protein [Homo sapiens]
22689	Hypothetical protein MGC2835 [Homo sapiens]
12247	BGAL_ECOLI Beta-galactosidase (Lactase)
119216	Heat shock protein HSP 90-alpha (HSP 86)
27753	2119399A elongin B [Homo sapiens]
73738	BGAL_ECOLI Beta-galactosidase (Lactase)
73716	I37273 highly charged protein
18093	JC5594 jerky gene protein homolog
2173	Ubiquitin-like, containing PHD and RING finger domains,
98457	Hypothetical protein LQFBS-1
7210	Hypothetical protein FLJ10330 [Homo sapiens]
12638	Hypothetical protein MGC2663 [Homo sapiens]
17190	M2A1_HUMAN Alpha-mannosidase II
3062	Ras-related protein Rab-43
6374	cDNA clone
24047	cDNA clone
15903	Rho-interacting protein 3
35112	CD262
3591	Nicotinamide nucleotide transhydrogenase
18885	SKI-like
53944	cDNA clone
35247	Zinc finger protein 367
12133	cDNA clone
11607	Bromodomain adjacent to zinc finger domain, 2A
92141	cDNA clone
39144	cDNA clone
21406	Trio: Triple functional domain (PTPRF interacting)
122488	cDNA clone
12730	cDNA clone
46347	Zinc Finger Protein 618

12316	cDNA clone
39271	Glyceronephosphate O-acyltransferase (Gnpat)
24426	Solute carrier family 39 member 10
60107	Zinc finger, DHHC domain containing 21
19162	CG4768-PA (predicted) (RGD1309748_predicted)
16328	Hypothetical protein (RGD1562788)
22883	cDNA clone
19457	cDNA clone
1207	Olfactory receptor Olr1558
6495	cDNA clone
49716	cDNA clone
104274	GI:13385412-like protein splice form I
22598	Transcription factor 1
1292	Tyrosine 3-monooxygenase/ activation protein
2862	Calpain-6
10495	LTC4 transporter
251774	Melanoma-associated antigen MG50
22518	Caveolin-1
2875	Collagen alpha 2 type V
22164	ADAM metallopeptidase with thrombospondin type 1 motif, 4
Mmu.6701	Myosin regulatory light chain 2-B
154399	STAT5A
166161	Coiled-coil domain containing 64
197185	60S ribosomal protein L21
28146	Diphosphomevalonate decarboxylase
3997	proteasome (Psma3)
N/A	Homeobox protein cut-like 1 (CDP1)
14280	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
218100	Zinc finger protein basonuclin-2
32641	F-box and leucine-rich repeat protein 2
101908	SERTA domain containing 4

Table 3.3 Unigene IDs and gene descriptions for the total number of Intuitive Transcripts. The tag frequencies in the VMP and VSU libraries are shown to give an approximation of transcript abundance. Genes with (PS) had only partial similarity to a known putative gene.

Unigene	VMP	VSU	Description
25124	6	0	Insulin like Growth Factor 1 (Ilgf1)
964	124	34	Insulin like Growth Factor 2 (Ilgf2)
37338	14	4	Latent transforming growth factor binding protein 3 (Ltbp3)
7961	49	15	Latent transforming growth factor binding protein 4 (Ltbp4)
8672	10	1	Tuskushi
41133	10	2	Sortilin-related VPS10 domain containing receptor 2 (Sorcs2)
8257	11	1	Semaphorin6D
11567	5	0	Neural epidermal growth factor-like 2 (Nell2)
106103	362	258	Decorin
98989	236	142	Secreted protein, acidic, cysteine-rich (SPARC)
22767	8	1	Sprouty1
15322	11	2	CKLF-like MARVEL transmembrane domain containing 3 (cmtm3)
3733	6	2	CD164
64490	12	0	ADAM metallopeptidase with thrombospondin type 1 motif, 4 (ADAM-TS4)
15864	6	1	Fc fragment of IgG, receptor, transporter, alpha (FCGRT)
34782	6	0	Wingless-related MMTV integration site 4 (Wnt4)
2269	18	5	Basigin

3.2 Results

3.2.1 General Outcome

A summary of the WISH analysis is provided in Table 3.4. From all three groups combined, 193 transcripts were available for WISH analysis. 53 transcripts of the 193 were selected to be analysed from all three groups by WISH because those mRNAs had definite gene identities. Of the 53 transcripts with definite gene identity, 23 failed at the PCR, cDNA isolation or *in vitro* transcription stages of the RNA probe production protocol. From the 30 remaining transcripts that were analysed by WISH, 13 mRNAs were expressed in the mesenchyme.

Table 3.4 Summary of the WISH analysis to confirm the transcripts stroma enriched status

Three groups were chosen for the analysis either based on the statistical analysis of the SAGE data or whether the gene product was a secreted or membrane-associated protein.

	Groups		
	Statistically selected	Non-Statistically selected	Intuitive
Total Number of Transcripts	54	122	17
Transcripts to be analysed	10	28	15
Riboprobe production failure	4	16	3
No stromal expression	4	8	5
Stromal expression	2	4	7

Various production issues arose during the RNA probe production process. For example, for all the transcripts of all three groups to be analysed, 194 primer pairs were required. Other stages of the RNA probe production method presented difficulties, such as the cloning of the PCR products into the pCR4 plasmid and the cloned product's subsequent isolation. Additionally, the *in vitro* transcription reaction sometimes failed to produce the appropriate RNA probe. Essentially, it was the coupling of a problematic RNA probe production method, with a large-scale analysis, that resulted in the small number of candidates being determined as enriched in the mesenchyme.

Thirteen transcripts were identified as showing a mesenchymal expression pattern, and are candidate stromal mediators of prostate growth. The expression of these candidates was examined in both male and female UGTs in the WISH analysis. Both sexes were used to control for high (male) and low (female) androgen environments. Additionally, the VMP represents a model of prostatic mesenchyme that does not contain epithelia. The prostatic epithelium in the male may affect the mesenchyme with regard to the expression of the transcripts in the three groups. Therefore it was important to examine the potential mesenchyme expression of the transcripts in both sexes.

3.2.2 Positive Control Transcripts

Prior to the start of this analysis, four transcripts from the 219 list had been determined as expressed in the mesenchyme by WISH. These transcripts were used as positive controls for this WISH analysis. The positive control transcripts used were Slit2, Robo1, Scube1 and Notch2 (Figure 3.2). At least one of these transcripts was used for every individual WISH experiment.

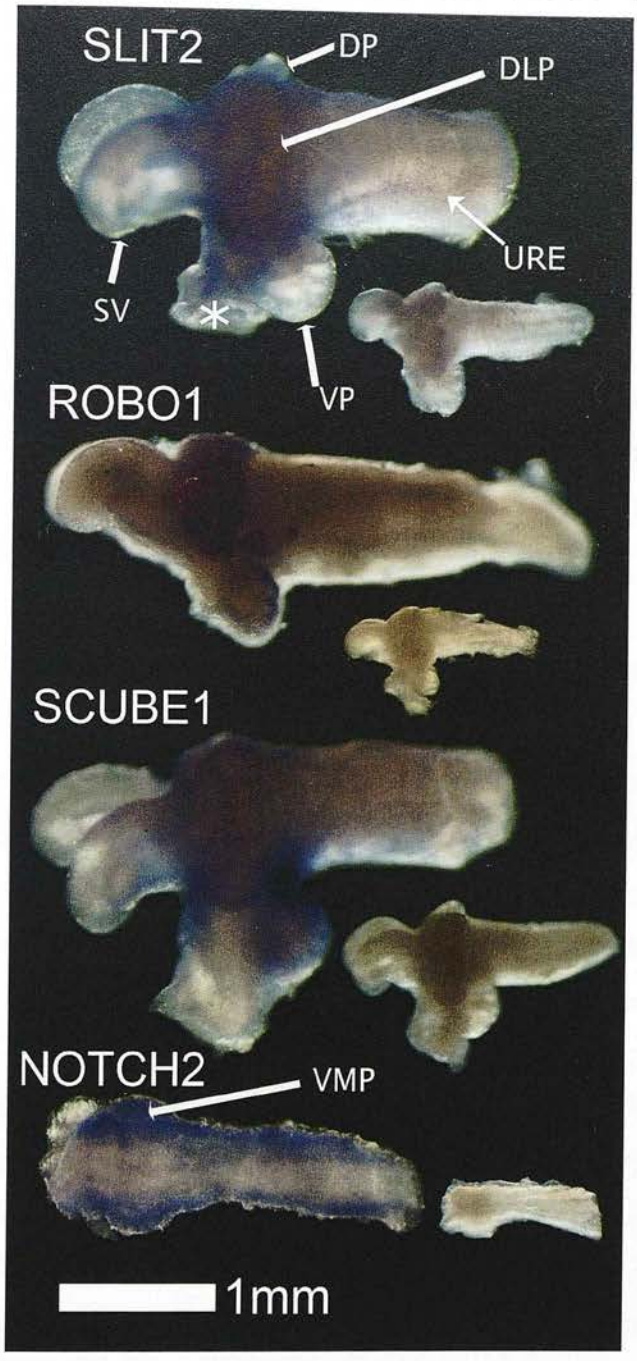

Figure 3.2 Positive controls for the WISH analysis. Slit2, Robo1 and Scube1 transcript expression are shown in d0 male rat UGTs. The expression of each control transcript can be seen in the mesenchyme of the dorsal-lateral and ventral prostate lobes. Notch2 expression is shown in a d0 female rat UGT. The expression of the Notch2 mRNA can be observed in the VMP and in the smooth muscle layer running surrounding the urethra. The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown to illustrate a negative signal and potential background staining. At least one of these

transcripts was used in each WISH experiment. (DP=Dorsal Prostate; DLP=Dorsal-Lateral Prostate; URE=Urethra; SV=Seminal Vesicles; VP=Ventral Prostate; VMP=Ventral Mesenchymal Pad). The asterisk shows the position where the bladder was removed.

The aim of the WISH analysis was to identify mRNAs expressed in the mesenchyme. An epithelial control to show any prostatic epithelial transcript expression was not included, which was a limitation of the WISH analysis. RNA probes were designed for a number of known prostatic epithelial markers including Nkx3.1, FoxA2, Sox9 and E-cadherin. None of the epithelial RNA probes provided epithelial specific expression. The reason for this was not determined but may be that epithelial specific probes require more permeable specimens.

3.2.3 WISH analysis of the Statistically Selected Transcript Group

There were 54 transcripts in the statistically selected group. 19 transcripts of the 54 had only a partial or no similarity to an Ensembl file. If the transcript did not have reliable sequence information, it would make it difficult to perform further characterisation. Therefore, any transcript with no reliable Ensembl data file was omitted from the WISH analysis. From 35 transcripts, the first 10 transcripts in the group were selected for RNA probe production. Of these 10, one transcript failed at the PCR stage, two failed at the specific cDNA isolation stage and one failed at the *in vitro* transcription stage of the RNA probe production protocol. Two transcripts did not produce a stromal expression pattern. Two transcripts from the 54 in total produced a definite stromal expression pattern. The two stromal transcripts identified were the Ras GTPase, RRAD/RasD (Caldwell et al, 1996) and tyrosine kinase receptor, EphB3 (Bohme et al., 1993).

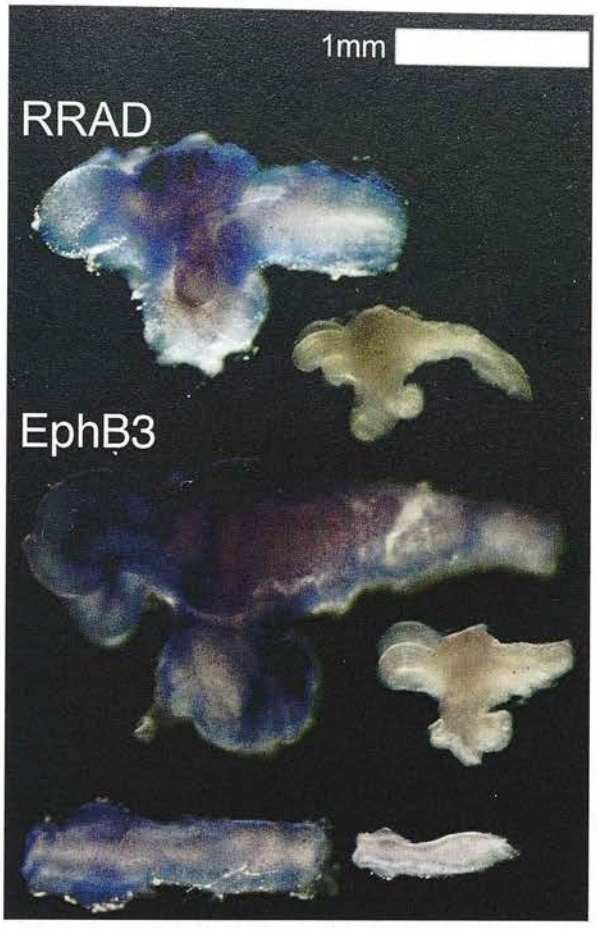

Figure 3.3. WISH Identification of 2 transcripts from the Statistically Selected Group expressed in the stroma.
RRAD/RasD, a Ras related GTPase, and the receptor tyrosine kinase, EphB3, are restricted to the mesenchyme in d0 rat UGTs (n=3). EphB3 is expressed, predominantly in the stroma, of all the visible prostate lobes and the SV. RRAD/RasD is expressed in the stroma of the DLP and to a lesser extent the VP The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown to illustrate a negative staining pattern and potential background staining.

RRAD/RasD is an intracellular GTPase that is a member of the Ras super family, which includes other GTPases families, Rho and Ral, involved in many intracellular signalling activities (Shou et al, 1992). RRAD/RasD also interacts with the calcium influx regulator, calmodulin that suggests it is involved in a variety of signalling roles. Calcium is one of the most common signalling transducers (Chang, et al., 2007).

EphB3 is involved in cell-to-cell signalling. EphB3 is a transmembrane receptor and 1 of 5 members of the EphB receptor tyrosine kinase family. The best characterised function of the EphB3 receptor is as a chemotactic guidance cue, during the development of the nervous system (Jevince et al., 2006). It has additionally has been implemented as being involved in organ development (Pickles et al., 2002) and cancer (Cortina et al., 2007). There is evidence that suggests a functional association between EphB receptors and the Ras super family. Co-expression of RasD and EphB3 may suggest a link between them which is discussed later in this chapter. EphB3 was chosen for further analysis and led to the EphB family becoming the focus of this thesis.

3.2.4 WISH analysis of the Non-Statistically Selected Transcript Group

There were 122 transcripts in the non-statistically selected group. Seventy transcripts of the 122 had a partial or no similarity to an Ensembl file. Of the remaining 52 transcripts, the first 28 transcripts in the group were selected for RNA probe production. From the 28, ten transcripts failed at the PCR stage and 6 failed at the specific cDNA isolation stage of the RNA probe production protocol. Eight transcripts did not produce a stromal expression pattern. Four transcripts from the 122 in total produced a stromal expression pattern.

Four transcripts from the non-statistically group were determined as mesenchyme enriched by the WISH analysis. These were ETL, CRIP2, LAT3, and RBMS1 (Figure 3.5). ETL is a seven transmembrane receptor and a member of the secretin family (Nechiporuk et al., 2001). CRIP2 is a zinc finger based transcription factor (Okano et al., 1993). LAT3 is a transmembrane amino acid transporter that is upregulated in prostate cancer as determined by differential display analysis (Colea et al., 1998). RBMS1 is an RNA binding protein (Negishi et al., 1994). These 4 proteins are not well characterised in terms of function in the prostate. The purpose of the non-statistically significant transcript group was as a control to the statistically significant transcript group. These two groups were designed to determine the efficiency of the SAGE analysis in identifying stromal specific transcripts. 4 non-statistically significant transcripts were

identified as stromal and 2 statistically significant transcripts were identified as stromal. This result suggests that statistics were not a completely reliable method of identifying transcripts expressed in the stroma.

In the male UGT, the expression of these transcripts was predominantly in the DLP. Only ETL and RBMS1 were expressed in the VP. CRIP2 and LAT3 both had no expression in the VP but show expression in the VMP (Figure 3.4 and 3.5). The differential expression between the male and female UGTs may be to be due to the effect of the prostatic epithelial in the male UGTs or androgens. For example, CRIP2 was expressed in the female VMP but not the male VP (Figure 3.4 and 3.5).

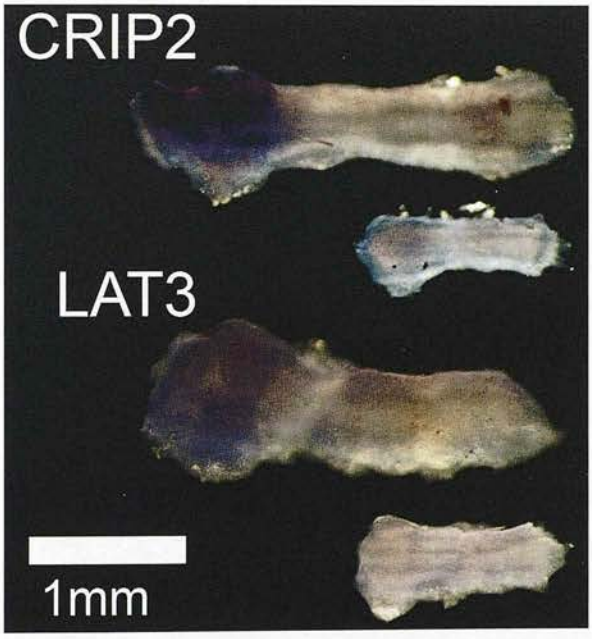

Figure 3.4 Stromal transcripts from the Non-Statistically Selected Group expressed in Female d0 UGTs. Only 2 of the 4 transcripts deemed stromal from this group were found to have any expression in the VMP (n=3). The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown to illustrate a negative signal and potential background staining.

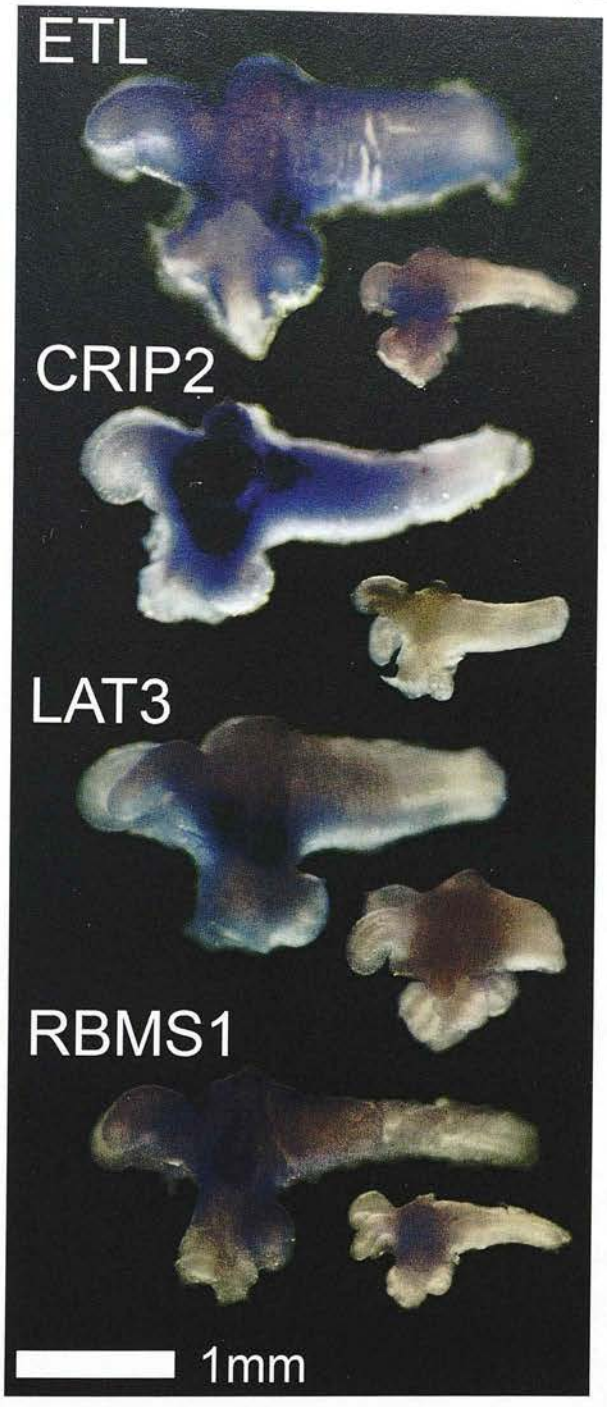

Figure 3.5 Stromal transcripts from the Non-Statistically Selected Group expressed in Male d0 UGTs. All 4 transcripts are expressed in the DLP. ETL, CRIP2 and RBMS1 are expressed in DP. ETL and RBMS1 are expressed in the VP. LAT3 expression is restricted to the peri-epithelial mesenchyme in the DLP. There is no transcript expression in the SV (n=3). The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown to illustrate a negative signal and potential background staining.

3.2.5 WISH analysis of the Intuitive Transcript Group

There were 18 transcripts in the intuitive group. Fifteen transcripts were randomly selected to be analysed. From the 15, two transcripts failed at the PCR stage and one failed at the specific cDNA isolation stage of the RNA probe production protocol. Five transcripts did not produce a stromal expression pattern. Seven transcripts from the 18 produced a stromal expression pattern. These 7 transcripts were Semaphorin 6D (Sema6D), Secreted Protein, Acidic, Cysteine-Rich (SPARC), Decorin, Tsukushi, Sprouty1, Sortilin-Related VPS10 domain Containing receptor 2 (Sorcs2), and Neural Epidermal growth factor-like 2 (NELL2).

Sema6D is a transmembrane receptor that has been characterised in chemotactic guidance (Qu et al., 2002). Both SPARC and Decorin are proteoglycans and known extracellular matrix components (Termine et al., 1981; Day et al., 1987). Tsukushi is a leucine rich secreted protein and a BMP inhibitor (Ohta et al., 2004). Sprouty1 is a membrane-associated protein and a FGF antagonist (Zhang et al., 2001). Sorcs2 is a VPS10 domain containing receptor protein of unknown function (Rezgaoui et al., 2001). NELL2 (neural epidermal growth factor-like like 2) is a secreted protein with many different functions (Watanabe et al., 1996).

Decorin, Sema6D and SPARC showed VMP expression, while Sema6D and SPARC showed expression in the SM layer (Figure 3.6). With the exception of SPARC, the other six intuitive transcripts showed DLP and VP expression. SPARC was restricted to the DLP (Figure 3.7)

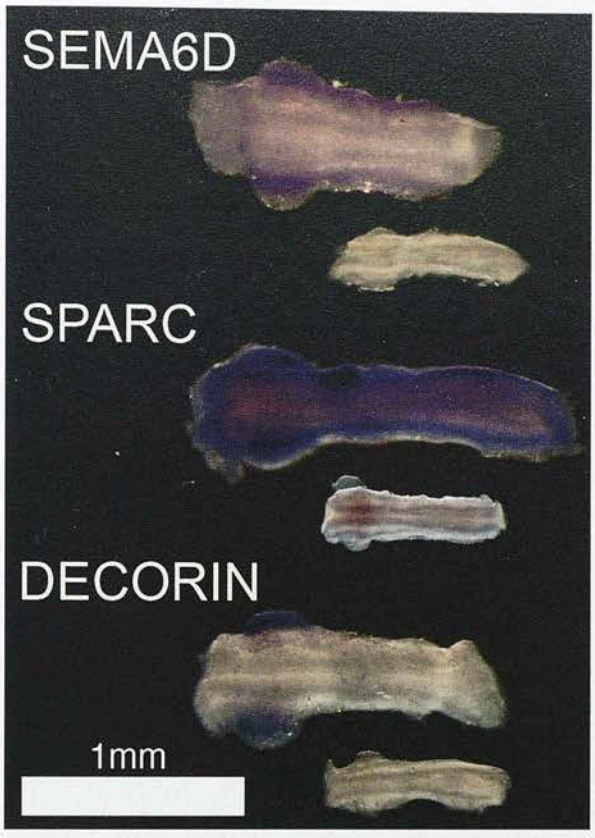

Figure 3.6 WISH analysis of transcripts from the Intuitive Group expressed in Female d0 UGTs. 3 of the 7 transcripts from the Intuitive Group showed stromal expression in d0 female UGT (n=3). The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are to illustrate a negative signal and potential background staining.

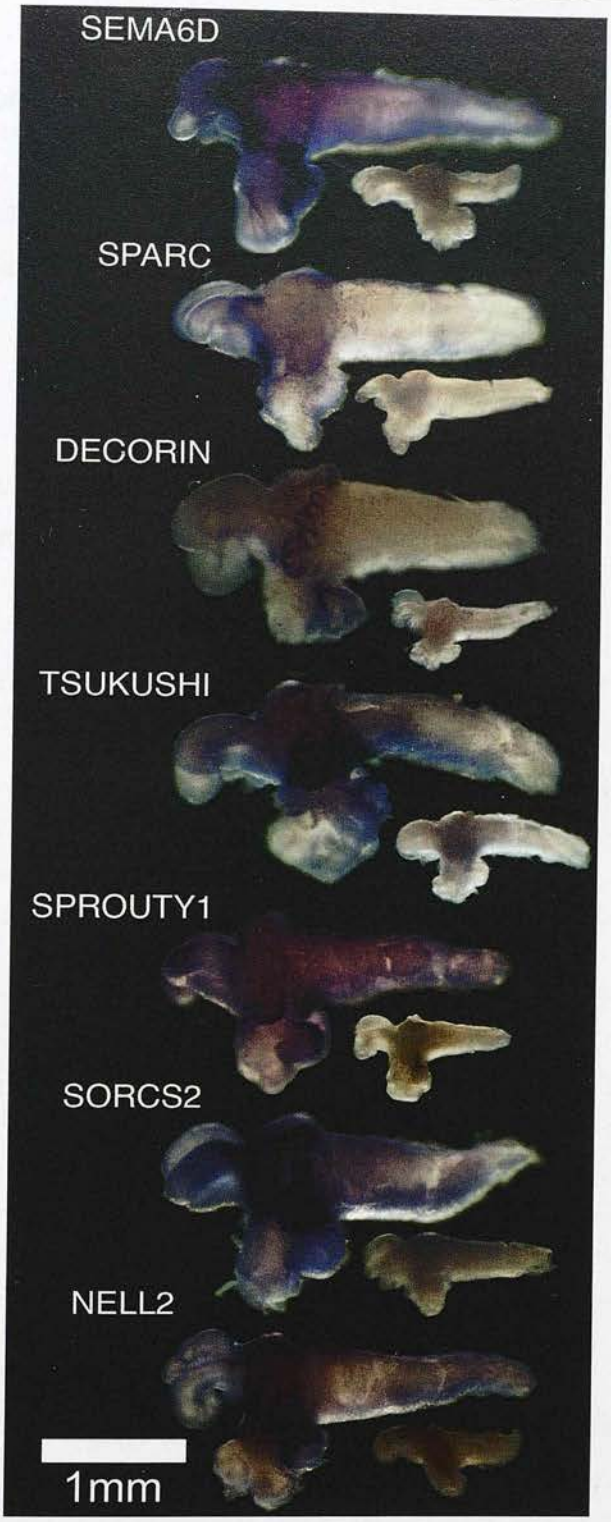

Figure 3.7 Stromal transcripts from the Intuitive Group expressed in Male d0 UGTs. 7 transcripts from the Intuitive Group were found to have stromal expression in Male d0 UGTs (n=3). The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown for to illustrate a negative signal and potential background staining.

3.2.6 Selection of Transcripts for Further Analysis

Thirteen transcripts were expressed in the stroma from the three groups. The full name, cellular location and known functions of these genes and their proteins are summarised in Table 3.5.

Table 3.5 Cellular location and functions of proteins encoded by the 13 stromal transcripts provided by the WISH analysis.

Gene	Description	Functions	Reference
Sema6D	Transmembrane	Various	Toyofuku et al; 2004
SPARC	Transmembrane	Various	Fukunaga-Kalabis and Herlyn 2007
Decorin	Extracellular	Various	Roughley and Lee; 1994
Tsukushi	Secreted	BMP inhibitor	Ohta et al; 2004
Sprouty1	Membrane	FGF inhibitor	Hanafusa et al; 2002
Sorcs2	Transmembrane	Neuropeptide signalling	Rezgaoui et al; 2001
Nell2	Secreted	Cell adhesion	Nelson et al; 2004
CRIP2	Cytoplasmic	Cell proliferation	Sun et al; 2008
LAT3	Transmembrane	Amino acid transport	Babu et al; 2003
ETL	Transmembrane	GPCR	Nechiporuk et al; 2001
RBMS1	Nucleus	Nucleotide binding	Negishi et al; 1994
RasD	Intracellular	Small GTPase	Zhu et al; 1995
EphB3	Transmembrane	Various	Adams et al; 1999

The goal of the WISH analysis was to identify mRNAs expressed in the stroma. A number of issues determined which candidate(s) should be analysed further in terms of their role, if any, in rat prostate development. Firstly, the amount of information already known about the candidate genes was one of the main criteria to consider. The more that is known about a particular gene, the easier is it to predict what role it could play in the development of the prostate. For example, a candidate may have a role in the development of another type of branching organ, such as the lung or kidney. This is

evidence that the candidate has a greater probability of being involved in the prostate development. Another advantage of an established gene in the literature is the greater number of gene specific research tools available. Such tools would include antibodies, recombinant proteins, small molecule inhibitors, and siRNAs.

The two mesenchyme-enriched transcripts considered for further were analysis were Sema6D and EphB3. Both genes encode cell-to-cell signalling receptors that have been characterised as molecular guidance cues for axons, which have a role in cellular positioning. Many Semaphorin family members have roles in organ development, such as the secreted protein Sema3A that inhibits ureteric bud branching morphogenesis (Tufro et al., 2008). However, Sema6D itself is less well researched. Sema6D has been suggested to be involved in axon guidance (Qu et al., 2002) and cardiac development (Toyofuku et al., 2004).

EphB3 has roles in a number of biological systems. These include roles in anorectal malformations (Yucel et al., 2007), urorectal development (Dravis et al., 2004) neural stem cell migration (Chumley et al., 2007), and colorectal cancer (Cortina et al., 2007) Additionally, EphB3 and its immediate family members and respective ligands have commercial antibodies and recombinant proteins available. No recombinant proteins were commercially available for either Sema6D or its putative ligand, PlexinA1 at the time this research was performed. Both EphB3 and Sema6D were expressed in the stroma of both male and female d0 UGTs. Therefore the data reported from the WISH analysis alone could not be used to distinguish which receptor protein should be characterized further in rat prostate development.

EphB3 was chosen for further investigation because it was it is a signaling receptor specifically expressed in the mesenchyme. Additionally, EphB3 had many research tools available which allowed expression and functional analysis in terms of rat prostate development.

3.3 Discussion

Three groups of transcripts were included in a WISH analysis to identify mRNAs expressed in the prostatic mesenchyme. The first group consisted of transcripts identified by statistics as enriched in the mesenchyme. The second group consisted of transcripts not identified by statistics as enriched in the mesenchyme. The rationale behind the selection of these two groups was that the WISH analysis would determine the efficiency of the SAGE analysis in identifying VMP specific transcripts. Additionally, a third group of transcripts which encode membrane proteins and secretory proteins was included as an intuitive group.

From 30 transcripts assayed across all three groups, 13 were determined as expressed in the mesenchyme. These 13 transcripts were candidates for further analysis. Nine transcripts identified as expressed in the mesenchyme were statistically significant transcripts, and four transcripts identified as expressed in the mesenchyme were non-statistically significant. As four non-statistically significant transcripts were identified as expressed in the mesenchyme, this suggests the statistics were not an absolute indicator of mesenchyme expression. Despite this, the number of candidates analysed was too small to definitely to determine if there was a real difference between the groups in terms of reliably predicting mesenchyme expression. The absence of a positive epithelial control was a limitation for the WISH analysis as it meant that epithelial expressed transcripts could not be identified. However, this limitation did not invalidate any stromal transcripts identified, though some transcripts identified as stromal may show both stromal and epithelial expression upon further analysis.

From the statistically selected group, two transcripts from the 54 transcripts produced a stromal expression pattern. The two stromal transcripts discovered were the Ras GTPase, RRAD/RasD (Caldwell et al, 1996) and tyrosine kinase receptor, EphB3 (Bohme et al., 1993). The RRAD/RasD is an intracellular signalling protein and the EphB3 receptor is involved in cell-to-cell signalling. EphB3 was selected for further analysis.

There is evidence to suggest that members of the Ras family, such as RRAD/RasD, are downstream intracellular mediators of EphB-EphrinB signalling. From studies using human umbilical vein endothelial cells, it has been proposed that EphrinB2-EphB interactions signal through the Ras/MAPK cascade (Kim et al., 2002). The colon cell line DLD1, that expresses the EphB2 receptor, responds to EphrinB1 stimulation by activating the small GTPases Rho and Ras (Riedl et al., 2005). This suggests an association between EphB signalling and Ras intracellular signalling mediators. Furthermore, the EphB2 receptor inhibits cell adhesion by phosphorylating the effector domain of R-Ras, As well as inhibiting R-Ras activity through phosphorylation, EphB receptors decrease R-Ras activity via a GTPase-activating protein (Dail et al., 2006).

There were 122 transcripts in the non-statistically selected group. Four transcripts from the 122 produced a stromal expression pattern. From the 4 transcripts, ETL and LAT3 are interesting candidates because ETL and LAT3 are cell surface proteins and may be involved in paracrine or juxtacrine cell signalling during prostate development. The non-statistically selected group of transcripts were included in the WISH analysis as a random control group. The function of the non-statistically selected group and statistically selected group of transcripts was to determine the efficiency of the SAGE analysis in identifying VMP specific transcripts. From the statistically selected group, two transcripts produced a stromal expression pattern. The WISH analysis identified 4 mRNAs that were not statistically significant in SAGE terms, but are expressed in the mesenchyme.

From the Intuitive Transcript Group, 7 transcripts were identified as stromal in either male or female UGT from the WISH analysis. The 7 transcripts came from the intuitive group, comprised of mRNAs that encode transmembrane and secreted proteins. More stromal transcripts were identified from the intuitive group than either of the other groups combined. So in terms of the identifying stromal mRNAs that are involved in signalling, the best criteria for selecting candidate mRNAs maybe the type of protein a particular transcript encodes or the cellular location of a particular encoded protein. These 7 transcripts make stromal candidates for further analysis. The intuitive group

provided a receptor (Sorcs2) and a secreted protein (Nell2) with little known about their functions. Additionally, the group provided extracellular proteins (SPARC and Decorin) and a receptor (Sema6D) that have been characterised in a number of biological systems and processes, but have not been explored in prostate development.

To summarise, 13 transcripts from the 194 mRNAs that comprise the three groups were identified stromal in either male or female rat d0 UGTs. Two of the transcripts, Sema6D and EphB3, were considered for further analysis. EphB3 was selected because this gene, its immediate family members and putative ligands were more thoroughly characterised in other biological models in the scientific literature. Furthermore, the EphB family had more analytical tools available. The rest of this thesis will cover the investigation of the EphB-EphrinB signalling family and its role, if any, in prostate development.

4 Expression of the EphB receptors and EphrinB ligands during Rat Prostate Development

4.1 Introduction

The WISH study identified expression of the EphB3 receptor in the VMP and prostate mesenchyme. The aim of this chapter was to determine which members of the EphB-EphrinB family were expressed during rat prostate development. 5 EphB receptors (EphB1, B2, B3, B4 and B6) and 3 Ephrin ligands (EphrinB1-B3) have been identified in mammals (Egea and Klein; 2007) EphB5 is not found in mammals but has been identified in the chicken (Soans et al., 1996). WISH, PCR and Immunohistochemistry were used to examine the expression of EphB receptors and EphrinB ligands at the mRNA and protein level during rat prostate development.

EphB receptors and EphrinB ligands are expressed in many biological systems. These include the brain (Willson et al., 2006), nervous system (Song et al., 2008) and blood vessels (Gale et al., 2001). EphB-EphrinB interactions have been shown to have a role in processes such as T-cell development (Alfaro et al., 2007) angiogenesis (Foo et al., 2006), and branching organ development such as the kidney (Ogawa et al., 2006). EphB3 and EphrinB1 were identified as expressed in the mesenchyme by SAGE and WISH analysis. PCR was used to investigate whether the other transcripts of the EphB and EphrinB families were expressed in the developing prostate. The PCR analysis was essential because SAGE may have missed the expression of other EphB and EphrinB transcripts, especially if they were expressed in the epithelium (see Table 4.1). Since we observed EphB3 and EphrinB1 expression in the developing prostate we hypothesised that EphB-EphrinB interactions may have a role in the rat prostate development.

Table 4.1 SAGE tag counts of EphrinB ligands and the EphB receptors. The EphrinB1 ligand and EphB3 receptor are the only members of the EphB-EphrinB family to be detected in the SAGE libraries. This data was taken from Vanpoucke et al 2007.

	VMP	VSU
EphrinB1	3	2
EphrinB2	0	0
EphrinB3	0	0
EphB1	0	0
EphB2	0	0
EphB3	5	0
EphB4	0	0
EphB6	0	0

.

4.2 Results

4.2.1 EphB and EphrinB mRNA Expression in d0 rat UGTs

PCR analysis was performed to determine which EphB and EphrinB mRNAs were expressed in male and female d0 UGT. VMP and VSU were included as these were the two tissues used in the SAGE analysis of prostatic transcript expression. Two primary rat cell types were also included. Ventral Mesenchyme Pad Cells (VMPC) are derived from the VMP of d0 female rat UGT, and Urogenital Stromal Cells (URSC) are derived from the SM and urethral mesenchyme from d0 female rat UGTs (Tomlinson et al., 2004). These primary cells were used as a comparison for the VMP and VSU tissues to help determine whether transcripts were expressed through out the stroma or particularly in VMP or SM compartments. From the literature, it is known that EphB and EphrinB are expressed in the rat brain and kidney (Willson et al., 2006, Ogawa et al., 2006). The lung, like the kidney, is a branching organ that has similar development mechanisms to the prostate. Lung, kidney and brain cDNA were used as positive controls. However, as

can be seen in Figure 4.1, the lung was an ineffective positive control. A no reverse transcriptase control from the VP cDNA synthesis was used as a negative control.

The PCR analysis of EphB and EphrinB expression is shown in Figure 4.1. The ligands, EphrinB1 and EphrinB2 were expressed in all the tissues except the lung. The EphrinB3 ligand was expressed in the brain and weakly expressed in the VMP and VSU. The EphB1 and EphB6 receptors were not expressed anywhere except in the brain. The EphB2 and EphB3 receptors were expressed in all tissues except the lung. The receptor EphB4 was weakly expressed in the VP, VMP and VSU. Both the EphB2 and B3 receptors and EphrinB1 and B2 ligands were observed in both the brain and the kidney. There are reports of EphB and EphrinB genes being expressed in the kidney (Takshashi et al., 2001) and the brain (Willson et al., 2006).

The receptors EphB2, EphB3, and ligands EphrinB1 and EphrinB2 were expressed in all prostatic tissues. These four genes were selected for further characterisation. EphB3 was not expressed in the VMPC and URSC primary cell cultures. This suggests the EphB3 expression may have been lost in primary cell culture, possibly due to a requirement for cell-to-cell contact, *in vivo* microenvironment or due to the selective growth of subpopulations *in vitro*. VMPCs and USRCs were not a good model for examining the role of EphB3 signalling in rat prostate development.

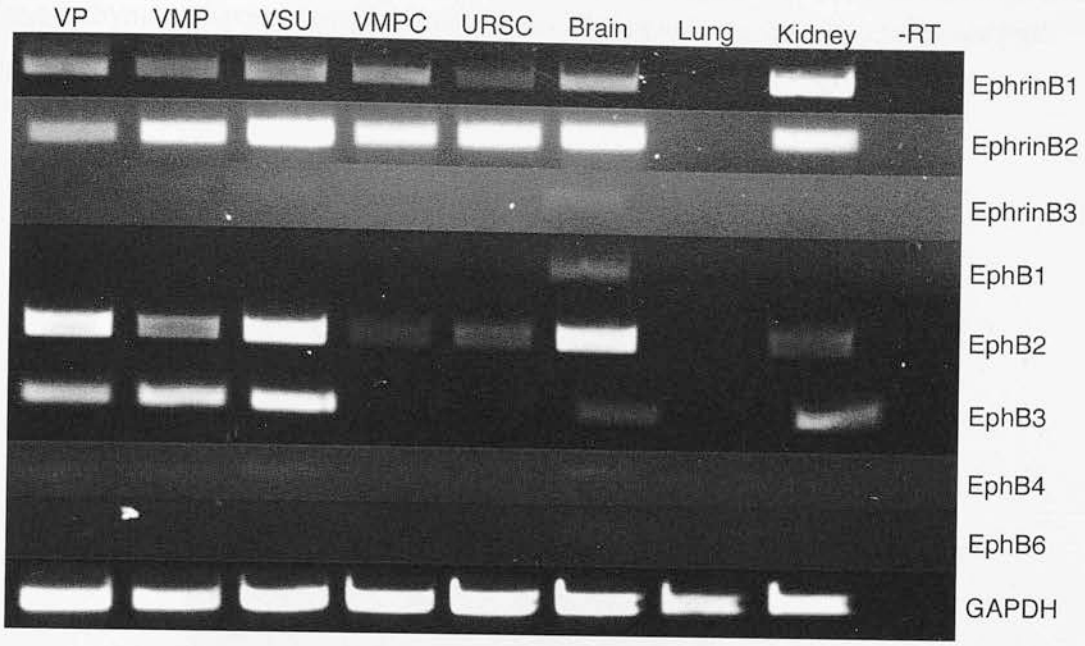

Figure 4.1. PCR analysis of EphB and EphrinB family members in d0 rat tissues. cDNA from the following tissues were used to establish whether the EphB and EphrinB mRNAs were expressed: Ventral Prostate (VP), Ventral Mesenchymal Pad (VMP), VMP, smooth muscle and urethral epithelium (VSU), two primary rat cell cultures; Ventral Mesenchymal Pad cells (VMPC) and Urethral Stromal cells (URSC). Brain, Lung and Kidney were included as positive controls. No Reverse Transcriptase (-RT) was included as a negative control (-RT). GAPDH was used as a loading control. PCR was repeated on three independent samples. For PCR product sizes, see Table 2.6 in the Methods and Materials.

Next, the expression of EphB2, EphrinB1, and EphrinB2 was examined by WISH to identify which might show mesenchymal expression and establish the spatial distribution. The EphB2 and EphrinB2 WISH did not show any stromal expression in either male or female d0 UGTs. These negative results may suggest that EphB2 and EphrinB2 are expressed in the epithelium. The EphrinB1 WISH result can be seen in Figure 4.2 which shows that EphrinB1 mRNA is expressed in the stroma. EphB3 and EphrinB1 are expressed in the mesenchyme of the DLP, VP and VMP. Therefore, a putative receptor (EphB3) and ligand (EphrinB1) binding pair are both expressed spatiotemporally in the prostate stroma. This results in a number of signalling possibilities. The EphrinB1 ligand may act on the epithelium, within

the mesenchyme or have a signalling role in both. The EphB3 receptor may receive signals from within the mesenchyme, from the epithelium or be involved in signalling from both.

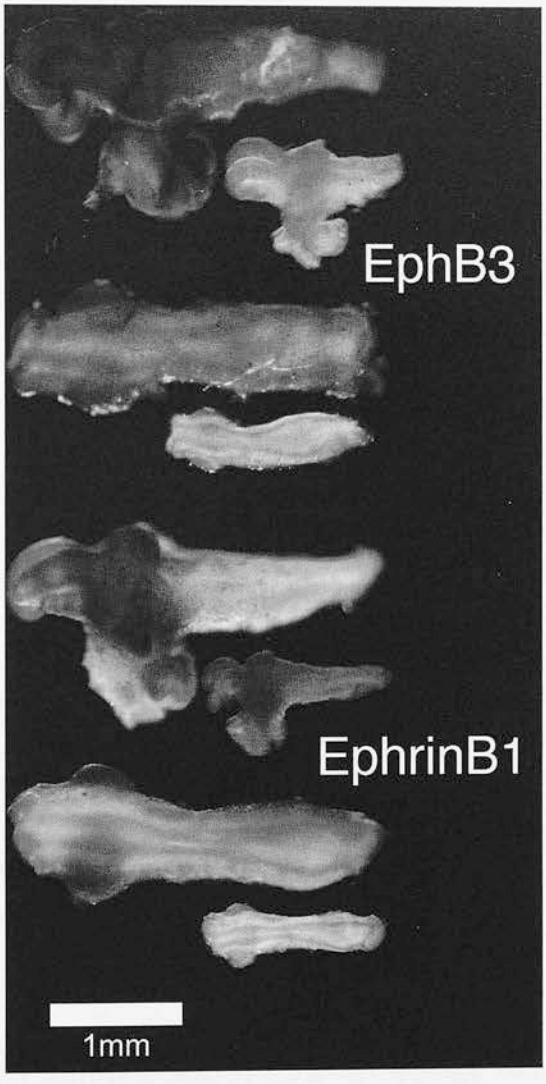

Figure 4.2. Expression of the EphB3 receptor and the ligand EphrinB1 in d0 male and female rat UGT. The EphB3 WISH is from the WISH analysis as described in Chapter 3. EphB3 and EphrinB1 are expressed in the mesenchyme of the VP and DLP on the males and the VMP in the females. The 1mm scale bar is for the larger antisense hybridised UGTs only. The sense UGTs are shown for control purposes (n=3)

4.2.2 mRNA Ontogeny of EphB receptors and EphrinB ligands in VP development

WISH analysis identified expression of EphB3 receptor mRNA and EphrinB1 ligand mRNA in the mesenchyme of d0 male and female rat urogenital tracts. The temporal expression of the receptors EphB2 and EphB3, and the ligands EphrinB1 and EphrinB2 was examined from e17 to adult (3 months) in the VP using quantitative PCR. The EphB2 receptor and EphrinB2 ligand were included in the quantitative PCR analysis as they were expressed in the VP, VMP and VSU (Figure 4.1).

EphB2 expression decreased from approximately 4-fold over TBP at e17 to approximately 2.5 fold over TBP by d0. This decrease in relative expression continued to approximately 1-fold over TBP by d10. The expression level of 1-fold over TBP continued until adulthood (Figure 4.3).

From e17 until d10, the EphB3 expression was steady at approximately 4-fold over TBP. However, at d35 EphB3 expression increased to 12.5 fold over TBP and then was reduced to approximately 5 fold over TBP by maturity (Figure 4.4).

EphrinB1 expression fluctuated between 6 and 8 fold over TBP between e17 and d10. There was then a reduction to approximately 1 fold over TBP by d35 followed by an increase to approximately 2.5 fold over TBP by adulthood (Figure 4.5).

EphrinB2 expression decreased from approximately 9 fold over TBP at e17 to approximately 4 fold over TBP by d0. This decrease in relative expression continued to approximately 1-fold over TBP by d35 and into adulthood (Figure 4.6).

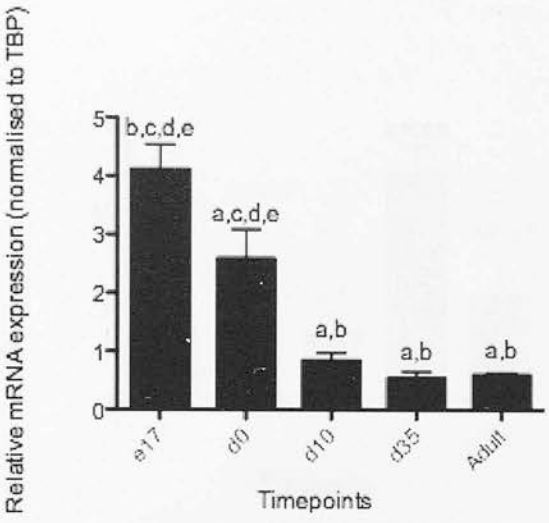

Figure 4.3 EphB2 Ontogeny during VP development. Expression of EphB2 decreased from the level at e17and there was no further decrease after d10. mRNA expression was normalised to TBP expression. "a" represents a statistically significance difference from e17, "b" represents a statistically significance difference from d0, "c" represents a statistically significant difference from d10, "d" represents a statistically significant difference from d35, "e" represents a statistically significant difference from Adult. These letters represent statistically significant differences. All the bars on each column represent the standard error for each time point. All statistical differences were calculated using Students t test at P = <0.05. All the data shown here represents the results from 3 independent experiments from 3 separate tissue extractions (n=3).

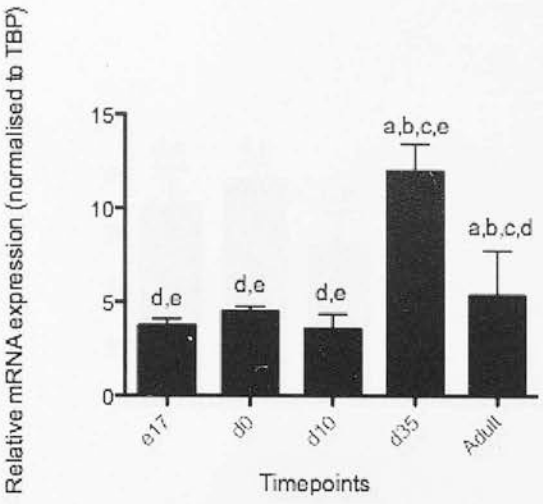

Figure 4.4 EphB3 Ontogeny during VP development. mRNA expression was normalised to TBP expression. Expression of EphB3 was similar at e17, d0 and d10 but showed a peak of expression at d35 that returned to levels similar to e17 by adulthood. "a" represents a statistically significance difference from e17, "b" represents a statistically significance difference from d0, "c" represents a statistically significant difference from d10, "d" represents a statistically significant difference from d35, "e" represents a statistically significant difference from Adult. These letters represent statistically significant differences. All the bars on each column represent the standard error for each time point. All statistical differences were calculated using Students t test at P = <0.05. All the data shown here represents the results from 3 independent experiments from 3 separate tissue extractions (n=3).

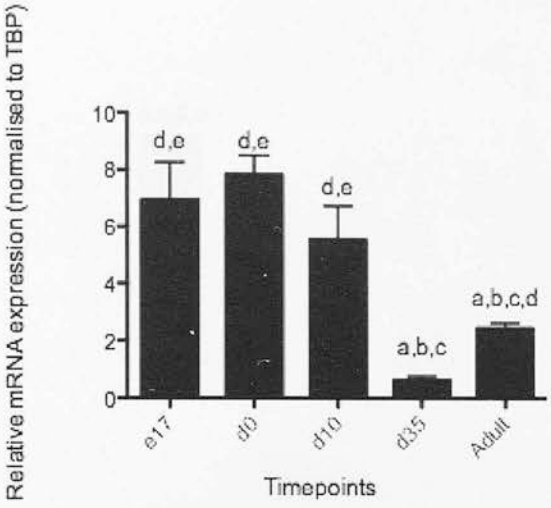

Figure 4.5 EphrinB1 Ontogeny during VP development. mRNA expression was normalised to TBP expression. EphrinB1 mRNA was abundant at e17 and expression remained high until d35 when EphrinB1 mRNA levels decreased rapidly but then increased again by adulthood "a" represents a statistically significance difference from e17, "b" represents a statistically significance difference from d0, "c" represents a statistically significant difference from d10, "d" represents a statistically significant difference from d35, "e" represents a statistically significant difference from Adult. These letters represent statistically significant differences. All the bars on each column represent the standard error for each time point. All statistical differences were calculated using Students t test at P = <0.05. All the data shown here represents the results from 3 independent experiments from 3 separate tissue extractions (n=3).

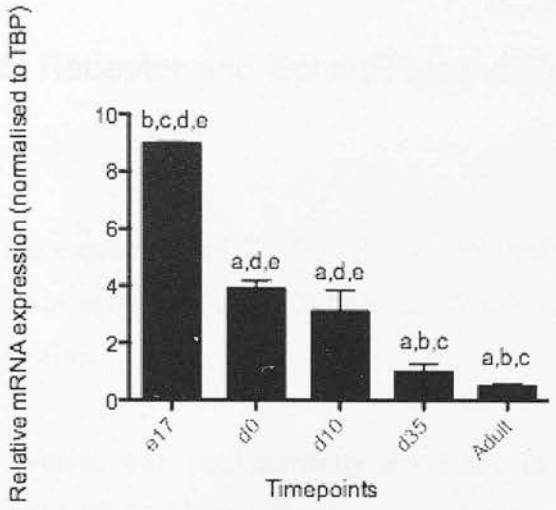

Figure 4.6 EphrinB2 Ontogeny during VP development. mRNA expression was normalised to TBP expression. Expression of EphB2 was high at e17 but by d0 was decreased and continues to decrease at d35 into adulthood. "a" represents a statistically significance difference from e17, "b" represents a statistically significance difference from d0, "c" represents a statistically significant difference from d10, "d" represents a statistically significant difference from d35, "e" represents a statistically significant difference from Adult. These letters represent statistically significant differences. All the bars on each column represent the standard error for each time point. All statistical differences were calculated using Students t test at P = <0.05. All the data shown here represents the results from 3 independent experiments from 3 separate tissue extractions (n=3).

4.2.3 EphB Receptor and EphrinB Ligand Protein Expression in d0 rat UGTs

To localise the expression of EphB2, EphB3, EphrinB1 and EphrinB2 proteins in the prostate, IHC was performed upon d0 male and female rat UGTs using antibodies specific for the four proteins.

The EphB3 protein was predominantly expressed in the stroma of the male UGT, particularly in the DLP and VP. There was very little EphB3 staining in the DLP and VP epithelium, suggestive of background staining (Figure 4.7). These observations are consistent with the WISH data for EphB3 (Figure 4.2).

In the d0 female UGT, EphB3 protein was expressed in the stroma and especially in the VMP and the SM layer (Figure 4.8). However, there was a weak level of EphB3 staining in the urethral epithelium in the female d0 UGT. The EphB3 staining in the urethral epithelium may be due to background staining. The SAGE data suggests that there was no EphB3 expression in the VSU because no EphB3 transcript tags were detected in the VSU library (Table 4.1). Furthermore, the WISH data showed no strong EphB3 transcript expression in the urethral epithelium (Figure 4.2).

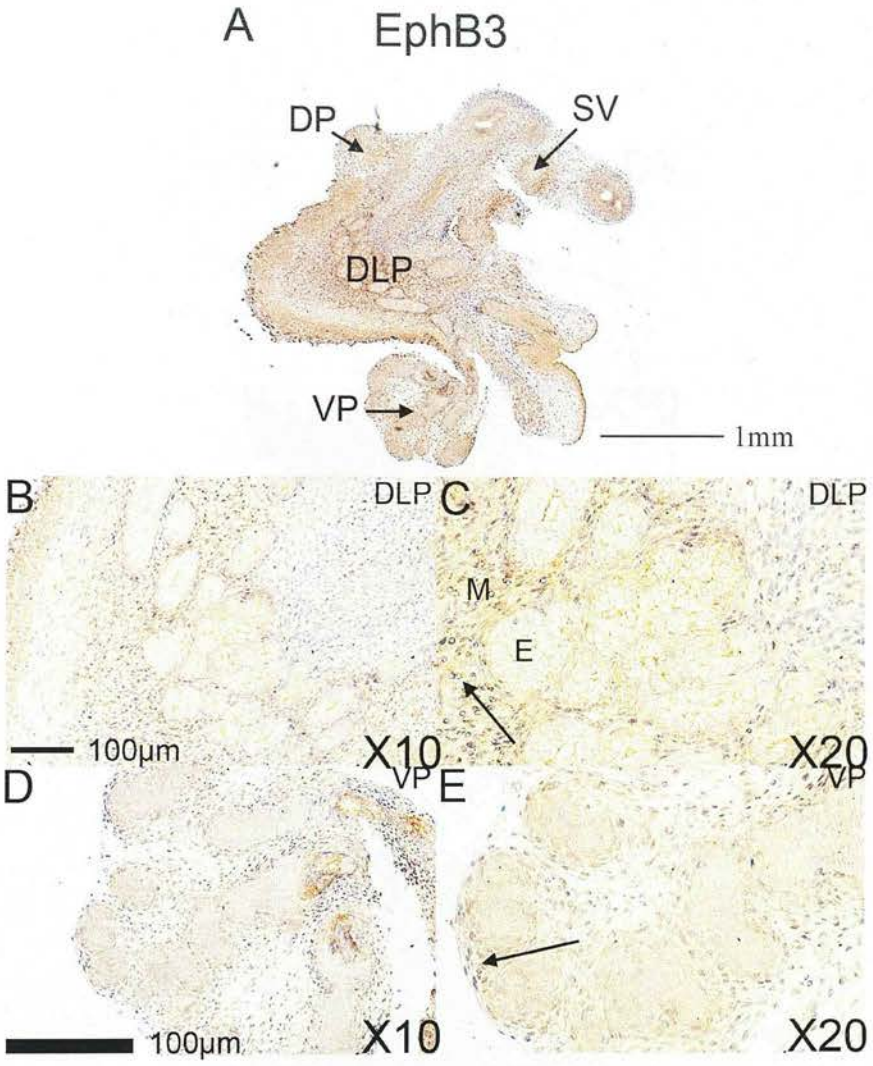

Figure 4.7 Expression of EphB3 protein in UGT of d0 male rat. A. d0 male UGT showing EphB3 staining in the mesenchyme. **B and C.** Magnified images of the DLP showing the mesenchyme expression of EphB3. The arrows indicate the peri-epithelial region. **D and E.** Magnified images of the VP showing the mesenchyme expression of EphB3. The arrows indicate the peri-epithelial region. DLP=Dorsal-Lateral Prostate, DP=Dorsal Prostate, E=Epithelium, M=Mesenchyme, SV=Seminal Vesicle, VP=Ventral Prostate (n=3).

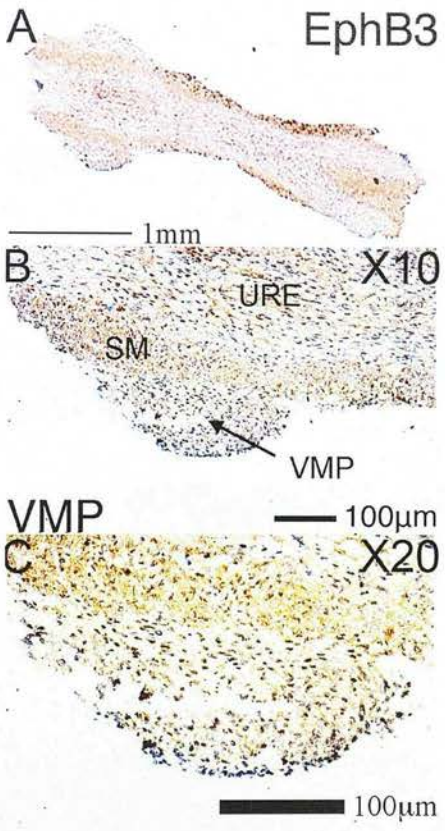

Figure 4.8 Expression of EphB3 protein in UGT of d0 female rat. A. d0 female UGT showing EphB3 staining in VMP, SM layer and urethral epithelium. **B and C.** Magnified images of the VMP and SM layer expression of EphB3. SM=Smooth Muscle, URE=Urethra, VMP=Ventral Mesenchymal Pad (n=3).

The EphB2 receptor protein was predominantly expressed in the prostatic epithelial buds in the VP, DLP and DP in the male d0 UGT. Conversely, the EphB2 protein was weakly expressed in the surrounding stroma, as seen in Figure 4.9. In the female UGT, EphB2 was expressed in the VMP, SM layer and the urethral epithelium (Figure 4.10). In contrast to the

EphB3 staining (Figure 4.8), the EphB2 staining in the urethral epithelium is likely to be genuine as no EphB2 transcript tags were detected in the SAGE data. This suggests EphB2 is expressed in the epithelium (Table 4.1).

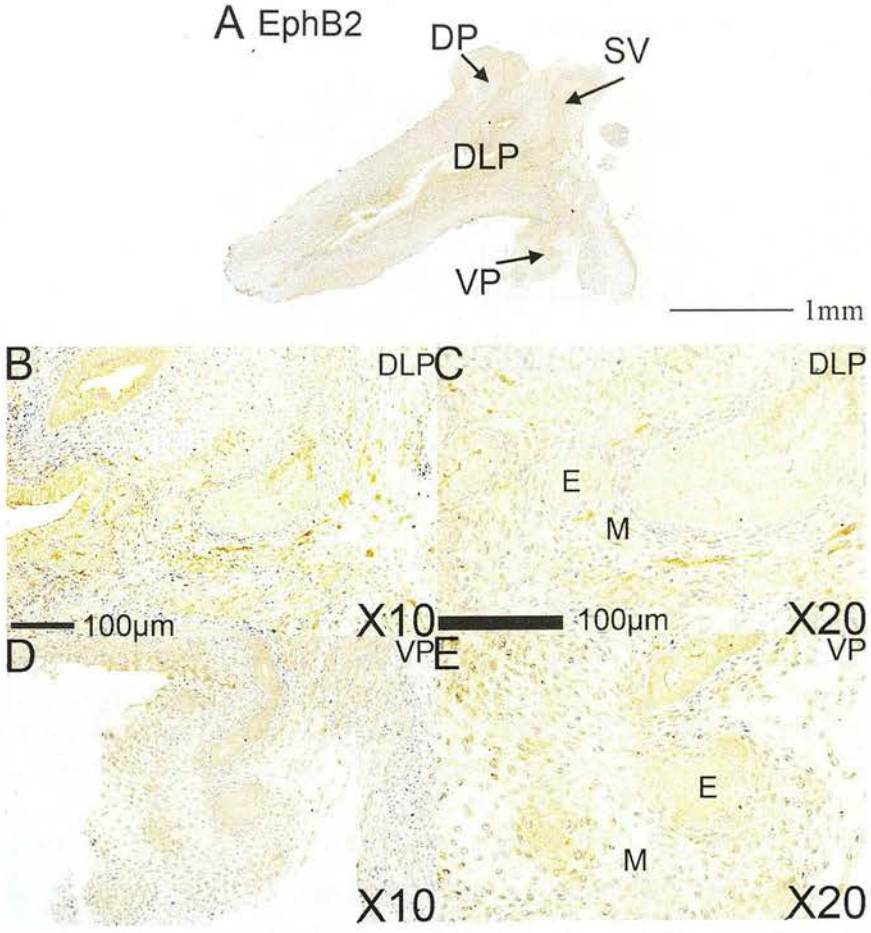

Figure 4.9 Expression of EphB2 protein in UGT of d0 male rat. A. d0 male UGT showing EphB2 staining in the in the epithelium. **B and C.** Magnified images of the DLP showing the epithelial expression of EphB2. **D and E.** Magnified images of the VP showing the epithelial expression of EphB2. DLP=Dorsal-Lateral Prostate, DP=Dorsal Prostate, E=Epithelium, M=Mesenchyme, SV=Seminal Vesicle, VP=Ventral Prostate (n=3).

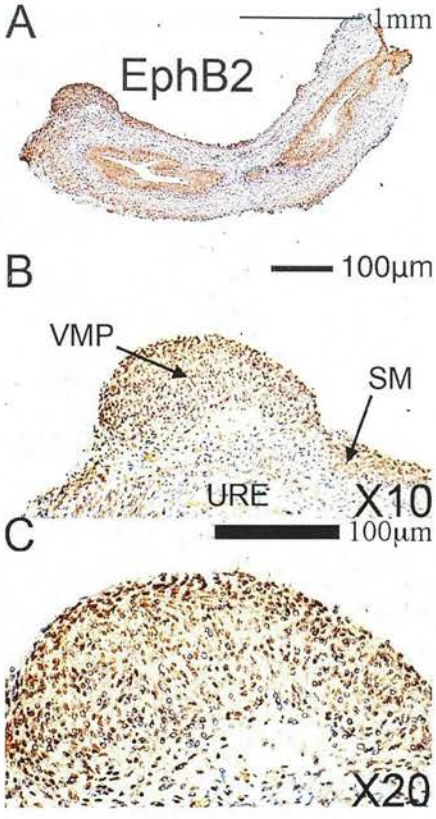

Figure 4.10 Expression of EphB2 protein in UGT of d0 female rat. A. d0 female UGT showing EphB2 staining in VMP, SM layer and urethral epithelium. **B and C.** Magnified images of the VMP and SM layer expression of EphB2. SM=Smooth Muscle, URE=Urethra, VMP=Ventral Mesenchymal Pad (n=3).

EphrinB1 was expressed in the mesenchyme in both male and female d0 UGTs. The expression of EphrinB1 was restricted to the peri-epithelial stroma surrounding the branching epithelial buds of all the prostatic lobes in the male. EphrinB1 was expressed in the peri-epithelial areas of the mesenchyme. Additionally, EphrinB1 expression was concentrated around the distal tips of the VP and DLP (Figure 4.11). In the female UGT, EphrinB1 was expressed in the VMP but was also expressed in the urethral epithelium (Figure 4.12). The EphrinB1 protein expression in the female urethral epithelium (Figure 4.10) is supported by the presence of two EphrinB1 SAGE tags in the VSU library

(Table 4.1). These two observations taken together suggest EphrinB1 is expressed in the female urethral epithelium. However, no EphrinB1 mRNA expression can be seen in the d0 female UGT using WISH (Figure 4.2). EphrinB1 protein was not expressed in either the urethral or prostate epithelium in the male d0 UGT (Figure 4.11).

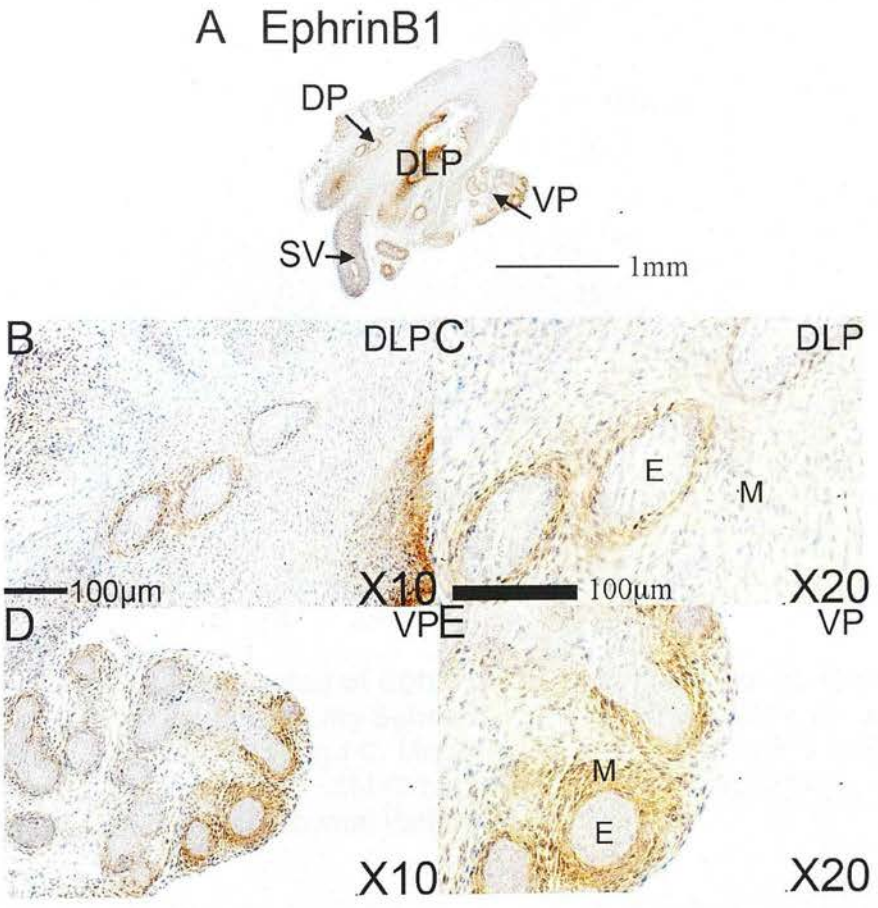

Figure 4.11 Expression of EphrinB1 protein in UGT of d0 male rat. A. d0 male UGT showing EphrinB1 staining in the in the peri-epithelial mesenchyme. **B and C.** Magnified images of the DLP showing the peri-epithelial mesenchyme expression of EphrinB1. **D and E.** Magnified images of the VP showing the peri-epithelial mesenchyme expression of EphrinB1 DLP=Dorsal-Lateral Prostate, DP=Dorsal Prostate, E=Epithelium, M=Mesenchyme, SV=Seminal Vesicle, VP=Ventral Prostate (n=3).

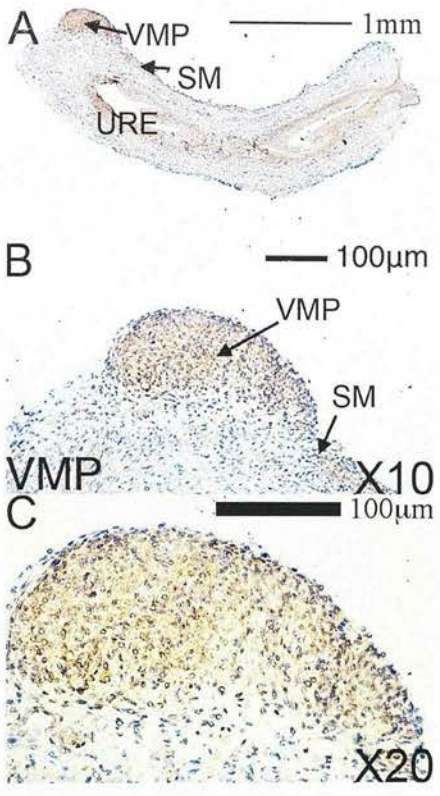

Figure 4.12 Expression of EphrinB1 protein in UGT of d0 female rat
A. d0 female UGT showing EphrinB1 staining in VMP, SM layer and urethral epithelium. **B and C.** Magnified images of the VMP and SM layer expression of EphrinB1. SM=Smooth Muscle, URE=Urethra, VMP=Ventral Mesenchymal Pad (n=3).

EphrinB2 was expressed in the VP and DLP epithelium of the male d0 UGT (Figure 4.13). In the female UGT, EphrinB2 was expressed in the VMP, SM layer and the urethral epithelium (Figure 4.14). The EphrinB2 staining in the urethral epithelium is likely to be genuine as no EphrinB2 transcript tags were detected in the SAGE data that suggests that EphrinB2 is expressed in the epithelium of the female UGT (Table 4.1).

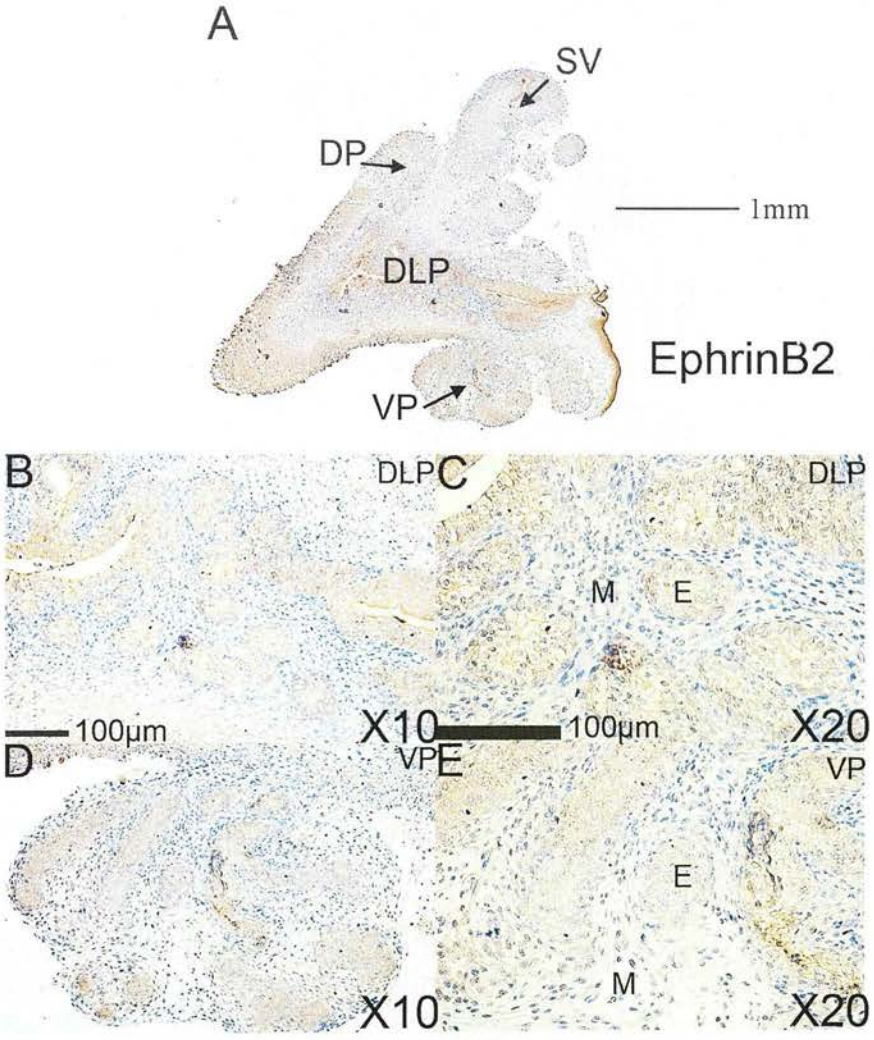

Figure 4.13 Expression of EphrinB2 protein in UGT of d0 male rat. A. d0 male UGT showing EphrinB2 staining in the in the epithelium. **B and C.** Magnified images of the DLP showing the epithelial expression of EphrinB2. **D and E.** Magnified images of the VP showing the epithelial expression of EphrinB2. DLP=Dorsal-Lateral Prostate, DP=Dorsal Prostate, E=Epithelium, M=Mesenchyme, SV=Seminal Vesicle, VP=Ventral Prostate (n=3).

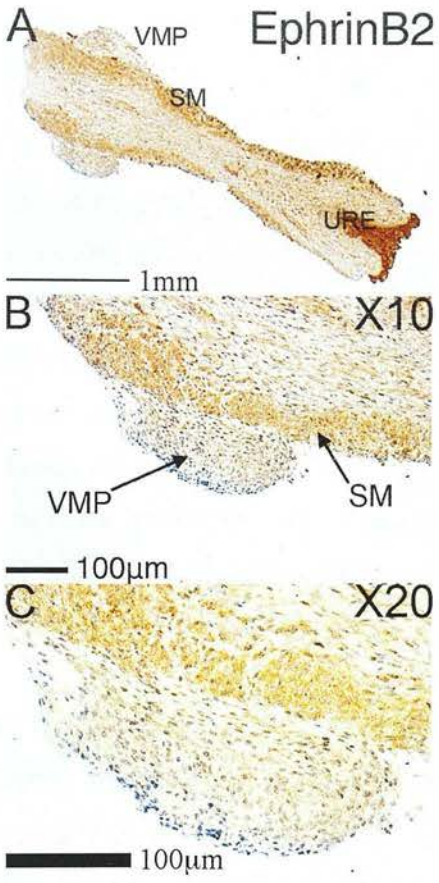

Figure 4.14 Expression of EphrinB2 protein in UGT of d0 female rat. **A.** d0 female UGT showing EphrinB2 staining in VMP, SM layer and urethral epithelium. **B and C.** Magnified images of the VMP and SM layer expression of EphrinB2. SM=Smooth Muscle, URE=Urethra, VMP=Ventral Mesenchymal Pad (n=3).

4.3 Discussion

The WISH analysis described in Chapter 3 identified EphB3 as expressed in the mesenchyme and lead to the analysis of the EphB family in the developing prostate. EphB3 has been characterised in other developmental systems and has a large number of specific research tools available. In mammals, the EphB family has 5 known receptors and 3 ligands. PCR analysis (Figure 4.1) revealed the receptors EphB2 and EphB3 and the ligands EphrinB1 and EphrinB2 were expressed in the rat prostate. The ligand EphrinB3 and the receptor EphB4 had very weak bands in the VP, VMP, and VSU lines, suggesting low expression levels or expression in small subset of cells. The receptor EphB1 was not expressed in any of the prostatic tissues (Figure 4.1). The EphrinB1 and EphrinB2 ligands and the EphB2 and B3 receptors were selected for further analysis.

The EphB3 transcript was detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the mesenchyme. WISH showed EphB3 as expressed in the VP and DLP mesenchyme of the male d0 urogenital tract. Additionally, EphB3 expression was restricted to the VMP in the female d0 urogenital tract (Figure 4.2). The EphB3 protein expression was consistent with the mRNA expression pattern and showed that EphB3 protein was expressed in the mesenchyme. However, there was a small level of EphB3 staining in the urethral epithelium in the female d0 UGT (Figure 4.8). The EphB3 staining in the urethral epithelium may of been non-specific staining. No EphB3 transcript tags were detected in the VSU SAGE library suggesting that there was no EphB3 expression in the VSU (Table 4.1). Furthermore, the WISH data showed no strong EphB3 transcript expression in the urethral epithelium (Figure 4.2). EphB3 is expressed predominantly in the mesenchymal tissues during chicken development (Baker et al., 2001).

EphB3 expression during rat VP development was approximately constant between e17 and postnatal d10 but increased by d35 before the levels decreased by adulthood (Figure 4.4). The increase in EphB3 mRNA levels at d35 is unusual when compared to the ontogeny of the other EphB-EphrinB family members examined in this thesis. The mRNA levels of

EphB2, EphrinB1 and EphrinB2 were approximately equal at d35 (Figures 4.3, 4.5 and 4.6). The EphB3 receptor may have an unknown ligand at d35 that is not one of the established EphrinB ligands. EphrinA ligands are known to interact with EphB receptors (Cerretti et al., 1995; Lackmann et al., 1997; Himanen et al., 2004). No investigation into the role of EphB3 signalling during VP development at d35 was conducted. Since EphB3 is expressed in the mesenchyme it may mediate signalling from Ephrins expressed in the epithelium, mesenchyme or both. The EphB3 expression data taken together suggests that EphB3 is involved in either paracrine signalling with the epithelium or has a role in intra-stromal autocrine / juxtacrine signalling during rat prostate development.

The EphB2 receptor protein was expressed in the male UGT epithelium (Figure 4.9) The EphB2 transcript was not detected by the SAGE analysis (Figure 4.1), which suggests epithelial expression as there was little epithelium in the SAGE libraries. These observations are in agreement with the epithelial specific expression of EphB2 in the developing ear epithelium (Cowan et al., 2000) and in vascular development (Salvucci et al., 2006).

EphB2 expression is sexually dimorphic in mouse genital tubercle development (Lorenzo et al., 2003). This suggests that EphB2 is a candidate androgen regulated gene in mouse genital development. However, the EphB2 receptor is expressed in both the male and female d0 UGTS (Figures 4.9 and 4.10) although this evidence is observational not quantitative. This may suggest that EphB2 is not an androgen-regulated gene in rat prostate development. The EphB2 mRNA expression during VP development (Figure 4.3) decreased between e17 and adulthood. This suggests any cell-to-cell signalling through the EphB2 receptor, expressed on either the stroma or epithelium, is occurring pre-natally. This is in an inverse correlation with androgen levels, further suggesting that EphB2 is not an androgen-regulated gene in rat prostate development.

EphrinB1 expression was restricted to the mesenchyme of the d0 male and female rat UGT at both the mRNA (Figure 4.2) and protein levels (Figures 4.11 and 4.12). In the d0 male UGT, EphrinB1 is expressed in the peri-epithelial mesenchyme. The EphrinB1 transcript

was detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the mesenchyme. EphrinB1 is expressed in the mesenchyme surrounding of other developing organs, such as the spinal cord (Jevince et al., 2006). Additionally, EphrinB1 is expressed in the mesenchyme during developmental processes such as corneal angiogenesis (Kojima et al., 2007). Collectively, this data suggests EphrinB1-EphB signalling is active during rat prostate development either in a paracrine (EphrinB1-EphB2), juxtacrine/autocrine (EphrinB1-EphB3) manner, or a combination of both.

The EphrinB1 expression pattern during VP development from e17 to adulthood (Figure 4.5) is similar to EphB2 (Figure 4.3). Both EphrinB1 and EphB2 mRNA expression decreased from e17 to adulthood. EphB2 was expressed in the epithelium and EphrinB1 expressed in the stroma. Collectively, these observations suggest that EphB2 and EphrinB1 interact in a paracrine fashion as a mechanism of stromal-epithelial interaction during rat VP development.

In the male d0 UGT, EphrinB2 was expressed in the epithelium (Figure 4.13). In the female UGT, EphrinB2 was predominantly expressed in the SM layer but was also expressed in the urethral epithelium and the VMP (Figure 4.14). The EphrinB2 transcript was not detected by the SAGE analysis (Figure 4.1), which suggests epithelial expression as there was little epithelium in the SAGE libraries. EphrinB2 was expressed in different tissue types in other developing biological systems. It is seen in the epithelium and mesenchyme during angiogenesis in the mouse (Adams et al., 1999; Korff et al., 2006). This data suggests that EphB-EphrinB juxtacrine signalling is active within the epithelium as EphrinB2 and the receptor EphB2 are expressed in the epithelium. Furthermore, EphrinB2 may interact with EphB3, expressed in the mesenchyme, creating a paracrine signalling mechanism between the mesenchyme and epithelium. The mRNA expression patterns of EphrinB2 (epithelium) and EphB3 (mesenchyme) were not similar during rat VP development. However, both EphrinB2 and EphB3 were expressed at relatively high levels from e17 to d10 postnatal. EphrinB2 mRNA expression decreased from e17 by maturity (Figure 4.6). This suggests any

juxtacrine or paracrine signalling involving EphrinB2 in VP development is important before d10.

To summarise, EphB3 and EphrinB1 were expressed in the prostate stroma whereas EphB2 and EphrinB2 were predominantly expressed in the prostate epithelium. Two paracrine mechanisms are possible. Firstly, signalling from receptor EphB2 (epithelium) to EphrinB1 (mesenchyme) is possible. Additionally, paracrine signalling from the receptor EphB3 (mesenchyme) to the ligand EphrinB2 (epithelium) is possible. Furthermore, juxtacrine signalling is possible between EphB3-EphrinB1 is possible in the mesenchyme and between EphB2-EphrinB2 in the epithelium. The expression patterns of these ligands and receptors during rat UGT development make it clear that EphB-EphrinB signalling has a role in rat prostate development. The next chapter attempts to elucidate the function of EphB-EphrinB signalling in rat prostate development.

5 Functional Analysis of the EphB Signalling in Rat Prostate Development

5.1. Introduction

The EphrinB1 ligand and EphB3 receptor were identified as expressed in the mesenchyme of the developing rat prostate. Additionally, the EphrinB2 ligand and EphB2 receptor appeared to be expressed in the epithelium of the developing rat prostate. EphB signalling is involved in the regulation of migration and cell adhesion during development. Processes influenced by EphB regulation include cell fate, morphogenesis and organogenesis (Merlos-Suarez and Batlle, 2008)

The aim of the research described in this chapter was to elucidate the function of EphB-EphrinB signalling in rat prostate development. The *in vitro* model used for this purpose was organ cultures of the VP (Sugimura et al., 1986; Lipshutz et al., 1997; Foster et al., 1999) and AP (Aboseif et al., 1997, Foster and Cunha., 1999) of d0 male rat prostate.

In vitro assays using the VP and AP cultures were performed. One assay used the EphrinB1-Fc and EphrinB2-Fc proteins whereas the other assay used the EphB2-Fc and EphB3-Fc proteins. The rationale for adding the EphB2-Fc and EphB3-Fc receptor proteins was that that they would competitively bind EphrinB ligands and inhibit the endogenous receptor-ligand interactions. This 'ligand trap' would result in a loss of Eph signalling activity. The receptor proteins were added as three separate treatment groups, EphB2-Fc, EphB3-Fc, and a combination of the two. The proteins were added at a concentration of 4μg/ml. Other studies have used much greater concentrations of EphB-Fc and EphrinB-Fc recombinant proteins in cultures such as at 5μg/ml in primary hippocampal neurone cultures (Hoogenraad et al., 2005) and 10μg/ml in in rat embryonic statacoustic ganglion cell culture (Bianchi and Gray; 2002) and mouse fetal

thymus organ culture (Alfaro et al., 2007). 4μg/ml was deemed sufficient for rat VP and AP organ culture. The unit of protein concentration, μg/ml, was used as opposed to molarity, as this was the conventional method of stating the protein concentration in the literature, as shown by the examples above.

The other assay used EphrinB1-Fc and EphrinB2-Fc proteins. The rationale for adding the EphrinB1-Fc and EphrinB2-Fc ligand recombinant proteins was that the added ligand would result in maximum levels of EphB signalling in the EphB receptor expressing cells. The ligand proteins were added as three separate treatments, EphrinB1-Fc, EphrinB2-Fc, and a combination of the two.

The assays were conducted in the presence and absence of testosterone (10^{-8} M). The concentration of testosterone was described in molarity as this is the convention in the literature (Sugimura et al., 1986; Freestone et al., 2003; Tomlinson et al., 2004). The presence of testosterone (T) in culture makes the assay more physiologically accurate. Any effect the EphB-Fc or EphrinB-Fc proteins may have on the organ culture may be masked by the presence of the T, hence the inclusion of the organ culture in the absence of testosterone. A control Fc treatment group was also included in every experiment to ensure that any biological effect on the organ was due to the Eph or Ephrin protein and not the Fc tag.

The effects of stimulation or inhibition of Ephrin signalling were measure in two ways. Firstly, the 2D area of each individual organ was measured after 6 days of culture (Sugimura et al., 1986; Lipshutz et al., 1997; Foster et al., 1999). The area measurement was used as an indicator of organ size. Secondly, the numbers of epithelial tips at the distal perimeter were counted and the distal perimeter itself was measured for each individual organ. Each tip count was divided by the perimeter distance. This resulting number was used to compare the degree of epithelial branching between each organ. These two different measurements, area and tip count, provided an insight into how the EphB-EphrinB proteins might affect organ growth and epithelial branching.

At the end of the organ culture period, the VP organs were treated with BrdU. Using fluorescent IHC, the amount of BrdU incorporated into proliferating cells was measured and used as a proliferation gauge in both stromal and epithelial cells. Co-staining with an epithelial marker enabled the calculation of epithelial and stromal proliferation rates. The stromal and epithelial cells were distinguished by using an anti-pan cytokeratin antibody to mark the epithelial cells. The number of total stromal and epithelial cells was counted in three random distal areas of ventral prostate for each organ. The total number of proliferating stromal and epithelial cells was calculated from these cell counts. All of the areas counted were adjacent to the edge of the organ. This ensured all the cell counts were representative of the distal part of each organ. The AP organs are much smaller than the VP organs, and were too small for reliable histology and IHC analysis of cell proliferation. Therefore it was only the VP organs that were used in the proliferation analysis and any other downstream IHC analyses.

VP grown *in vitro* and treated with EphB-Fc and EphrinB-Fc proteins were examined by histology and IHC to look at effect upon the epithelium and mesenchyme morphology and composition. Antibodies against specific marker proteins used for each tissue type. The basal epithelial marker p63 was used as a marker for epithelial differentiation. It is a transcription factor (Signoretti et al., 2000) and is expressed in undifferentiated epithelium at the growing tips of buds. Upon canalisation, p63 expression becomes restricted to the basal cells. Smooth Muscle Actin (SMA) is a stromal marker that was used as a marker for the smooth muscle surrounding the branching epithelia (Mitchell et al., 1990).

5.2 Results

5.2.1 The Effect of EphB-Fc Proteins on VP organs grown *in vitro*

The addition of the receptor protein, EphB2-Fc and EphB3-Fc, had an effect on the VP organs (Figure 5.1A). The addition of the receptor proteins decreased the area of VP organs grown in the presence of testosterone (+T+EphB2-Fc 34.7% decrease compared to +T, Students t test P=0.0057 at 95% confidence; +T+EphB3-Fc 26.3% decrease compared to +T, Students t test P=0.0108 at 95%) as shown in Figure 5.1B. This was expected, as if EphB signalling had a role in VP development, the exogenous EphB2-Fc and EphB3-Fc proteins would bind to and saturate the endogenous EphrinB ligands. This would result in the endogenous receptor proteins being unable to bind to the endogenous EphrinB ligands. The addition of both EphB2-Fc and EphB3-Fc together to a single culture produced no significant effect on VP size (Figure 5.1B). Exposure to EphB2-Fc induced a significant decrease in the degree of epithelial branching in VP organs (+T+EphB2-Fc 36% decrease compared to +T, Students t test P=0.0088 at 95% confidence.) as shown in Figure 5.1C. However, the treatments of EphB3-Fc and both proteins together had no significant effect on the degree of epithelial branching in VP organs (Figure 5.1C). It could be expected that a compound effect would be seen resulting in a greater decrease in area as both EphB2-Fc and EphB3-Fc might function additively. However, the presence of both EphB2-Fc and EphB3-Fc in culture had no effect on VP organ size.

No EphB-Fc receptor experiment produced a statistically significant result in the absence of testosterone. This could suggest that EphB signalling is androgen dependent during its mediation of VP development. However, the more likely explanation is that VP organs not exposed to testosterone during growth do not grow at a rate great enough for any effect of EphB signalling to be detected. Essentially, the assay involving the

EphB-Fc treatments is too insensitive enough to detect any detrimental effects on the VP in the absence of testosterone.

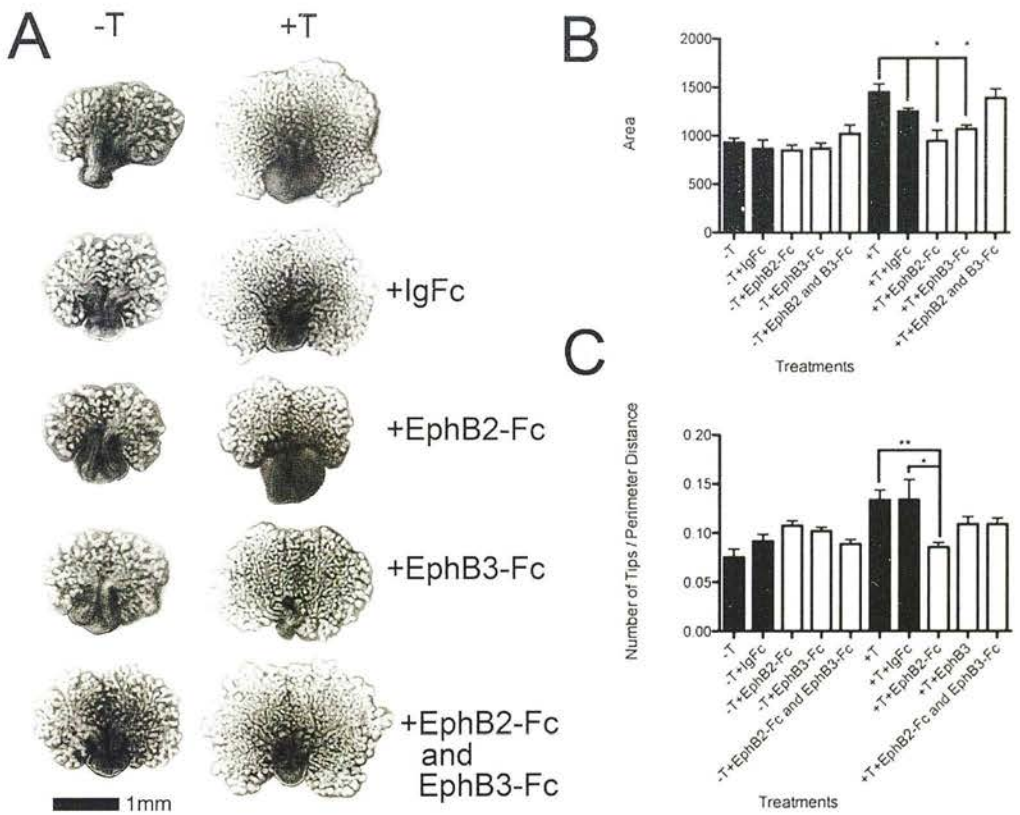

Figure 5.1. The Effect of EphB-Fc proteins on VP organ size and epithelial branching. Day 0 rat VP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M) and/or Fc control, EphB2-Fc, EphB3-Fc, or both (4µg/ml). The data represents a minimum of 2 organs from each treatment group from at least 3 independent experiments. All images taken at X4 magnification. Asteriks on graphs indicate statistical significance (* = $P<0.05$; **=$P<0.01$) **A.** Representative pictures of VP organ cultures. **B**. The 2D VP organ area was measured using Image J software from NIH. Area units were measured as pixels. Addition of EphB2-Fc and EphB3-Fc to VP organ cultures in presence of T resulted in a significant reduction in area (+T+EphB2-Fc 34.7% decrease compared to +T, Students t test $P=0.0057$ at 95% confidence; +T+EphB3-Fc 26.3% decrease compared to +T, Students t test $P=0.0108$ at 95%) confidence. **C**. The number of epithelial tips around the distal perimeter from each treatment were counted and displayed as the ratio of tip number to

perimeter distance. Addition of EphB2-Fc to VP organ cultures in the presence of T resulted in a significant reduction in epithelial tip number (+T+EphB2-Fc 36% decrease compared to +T, Students t test P=0.0088 at 95% confidence.)

5.2.2 The Effect of EphB-Fc Proteins on Cell Proliferation in the VP

The EphB2-Fc and EphB3-Fc treatments reduced VP organ size. Therefore, the proliferation rates of both epithelial and stromal cells in the VP were measured. The proliferation rates were measured using BrdU and pan-cytokeratin immunohistochemistry. The number of BrdU positive cells was counted in each tissue type from randomly selected areas of the VP periphery.

Representative fluorescent immunohistochemical images used in these experiments can be seen in Figure 5.2A. The addition of the EphB2-Fc and EphB3-Fc treatments had no significant effect on either VP epithelial or stromal proliferation when compared to the controls in the presence of testosterone (Figure 5.2B and C). In the absence of testosterone, no effect was observed on the number of proliferating epithelial cells. Conversely, the number of stromal cells in the VP organs treated with EphB2-Fc increased compared to the number of stromal cells of –T treated VP organs (-T+EphB2-Fc 35% increase compared to -T, Students t test P=0.0045 at 95% confidence) as shown in Figure 5.2C. There was a 35.9% increase in the number of proliferating epithelial cells between the +T+EphB2-Fc and the +T+IgFc control (Students t test P=0.0375 at 95% confidence). However, as there was no significant difference between the +T+EphB2 and +T control (Figure 5.2B). Additionally, There was a 54% increase in the number of proliferating stromal cells between +T+EphB2-Fc and +T treated organs (Students t test P=0.0272 at 95% confidence). However, as there was no significant difference between the +T+EphB2 and +T+Fc control (Figure 5.2C).

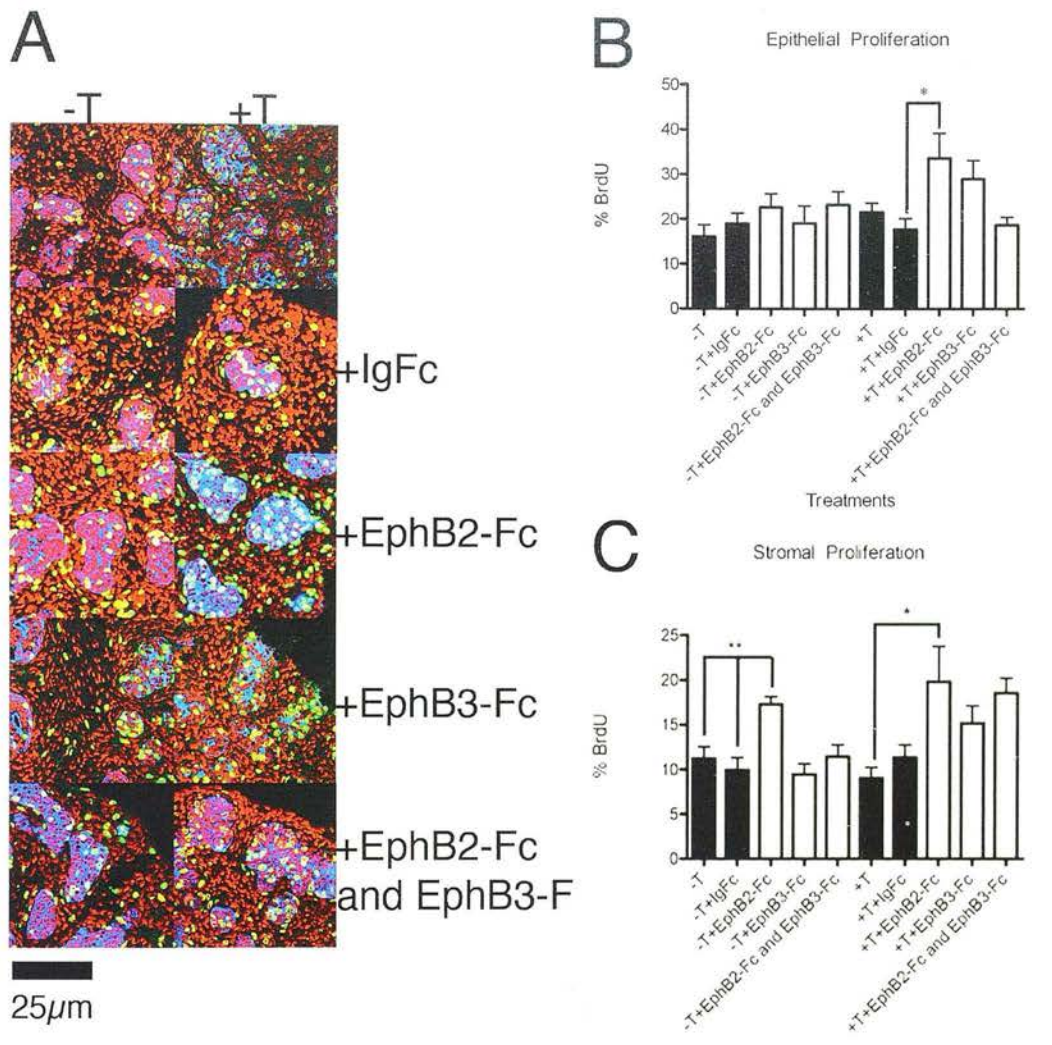

Figure 5.2. The Effect of EphB-Fc proteins on proliferation of epithelial and stromal cells in the distal region of VP organs cultured for 6 days. Day 0 rat VP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M) and/or Fc control, EphB2-Fc, EphB3-Fc, or EphB2-Fc and EphB3-Fc (4μg/ml). All images taken at X40 magnification. Asteriks on graphs indicate statistical significance (* = $P<0.05$; **=$P<0.01$) **A**. BrdU incorporation in distal regions to the urethra was detected by IHC (green, yellow when co-localised to nuclei) and epithelial cells were distinguished by IHC for pan-Cytokeratin (blue). Nuclei were counterstained with propidium iodide (red). **B**. Data representing epithelial cell proliferation taken from a minimum of 2 organs from each treatment group from at least 3 independent experiments. Addition of EphB2-Fc to VP organ cultures in the presence of T resulted in a significant increase in proliferation (-T+EphB2-Fc 44% increase compared to –T+IgFc, Students t test $P=0.0375$ at 95% confidence) **C**. Data representing stromal cell proliferation

taken from a minimum of 2 organs from each treatment group from at least 3 independent experiments. Addition of EphB2-Fc to VP organ cultures in the absence of T resulted in a significant increase in proliferation (-T+EphB2-Fc 35% increase compared to -T, Students t test P=0.0045 at 95% confidence). Addition of EphB2-Fc to VP organ cultures in the presence of T resulted in a significant increase in proliferation (-T+EphB2-Fc 36% increase compared to -T, Students t test P=0.0272 at 95% confidence).

5.2.3 The Effect of EphrinB-Fc Proteins on VP organs grown *in vitro*

The addition of EphrinB-Fc proteins to VP organ cultures resulted in a significant increase in the VP organ size in the presence of testosterone (+T+EphrinB1-Fc 24.2% increase compared to +T, Students t test P=0.0406 at 95% confidence; +T+EphrinB2-Fc 24.75% increase compared to +T, Students t test P=0.0309 at 95% confidence) as shown in Figure 5.3B. Addition of both ligands together did not increase organ size. No significant change in organ size was observed between when the EphrinB ligands were added in the absence of testosterone (Figure 5.3B).

Both EphrinB1-Fc and EphrinB2-Fc treatments resulted in a ~30% decrease in epithelial branching. Furthermore, the addition of both ligands together provided a 46% decrease in the degree of epithelial branching (+T+EphrinB1-Fc 32.7% decrease compared to +T, Students t test P=0.0127 at 95% confidence; +T+EphrinB2-Fc 29.5% decrease compared to +T, Students t test P=0.0347 at 95% confidence; +T+EphrinB1 and B2-Fc 46% decrease compared to +T, Student's t test P=0.0003) as shown in Figure 5.3C. No statistically significant differences were observed in the absence of testosterone. The overall effect of EphrinB-Fc protein treatment on VP organs was increased organ size and a decreased degree of epithelial branching. These observations may have been due to larger epithelial buds.

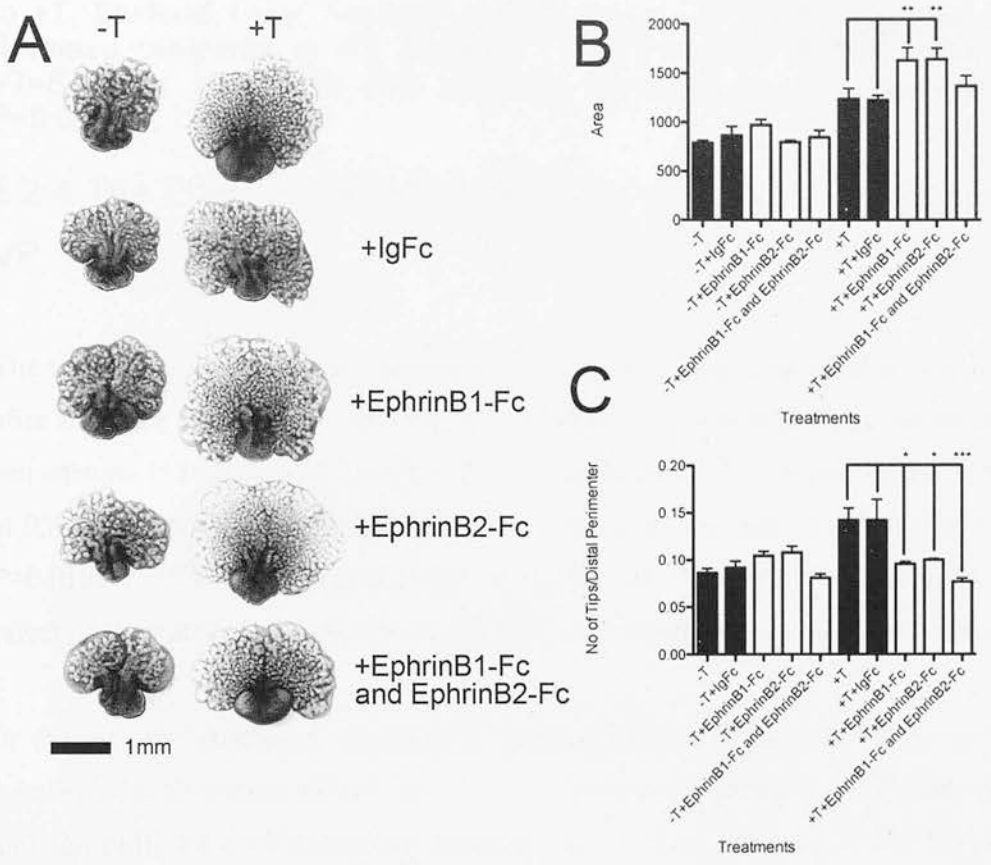

Figure 5.3. The effect of EphrinB-Fc Proteins on VP organ size and epithelial branching. Day 0 rat VP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M) and/or Fc control, EphrinB1-Fc, EphrinB2-Fc, or EphrinB1-Fc and EphrinB2-Fc (4µg/ml). Organs were imaged under 40X magnification using light microscopy before fixation. The data represents a minimum of 2 organs from each treatment group from at least 3 independent experiments. All images taken at X4 magnification. Asteriks on graphs indicate statistical significance (* = P<0.05; **=P<0.01, ***=P<0.001) **A.** Representative pictures of VP organ cultures. **B**. The 2D VP organ area was measured using Image J freeware from NIH. Area units were measured as pixels. Addition of EphrinB1-Fc and EphrinB2-Fc to VP organ cultures in the presence of T resulted in a significant increase in area (+T+EphrinB1-Fc 24.2% increase compared to +T, Students t test P=0.0406 at 95% confidence; +T+EphrinB2-Fc 24.75% increase compared to +T, Students t test P=0.0309 at 95% confidence. **C**. The number of epithelial tips around the distal perimeter from each treatment were counted and displayed as the ratio of tip number and perimeter distance. Addition of EphrinB1-Fc, EphrinB2-Fc, and EphrinB1 and B2-Fc together to VP organ cultures in presence of T resulted in a significant decrease in epithelial tip number (+T+EphrinB1-Fc 32.7% decrease compared

to +T, Students t test P=0.0127 at 95% confidence; +T+EphrinB2-Fc 29.5% decrease compared to +T, Students t test P=0.0347 at 95% confidence; +T=EphrinB1 and B2-Fc 46% decrease compared to +T, Student's t test P=0.0003).

5.2.4 The Effect of EphrinB-Fc Proteins on Cell Proliferation in the VP

The number of epithelial cells proliferating in the VP organ cultures increased by 28% after exposure to individual EphrinB1-Fc and EphrinB2-Fc treatments in the presence of testosterone (+T+EphrinB1 28.06% increase compared to +T, Student's t test P=0.0191 at 95% confidence; +T+EphrinB2-Fc 28.04% increase compared to +T, Student's t test P=0.0109 at 95% confidence) as shown in Figure 5.4B. The EphrinB-Fc proteins had no effect on the number of proliferating epithelial cells in the absence of testosterone.

In the VP, the combined treatment of EphrinB1-Fc and EphrinB2-Fc increased the number of proliferating stromal cells in the presence of testosterone (+T+EphrinB1-Fc and EphrinB2-Fc 48.6% increase compared to +T, Students t test P=0.0009 at 95% confidence) as shown in Figure 5.4C. Neither of the individual treatments of EphrinB-Fc produced a significant effect upon the number of proliferating stromal cells. The EphrinB-Fc proteins had no effect on the number of proliferating stromal cells in the absence of testosterone.

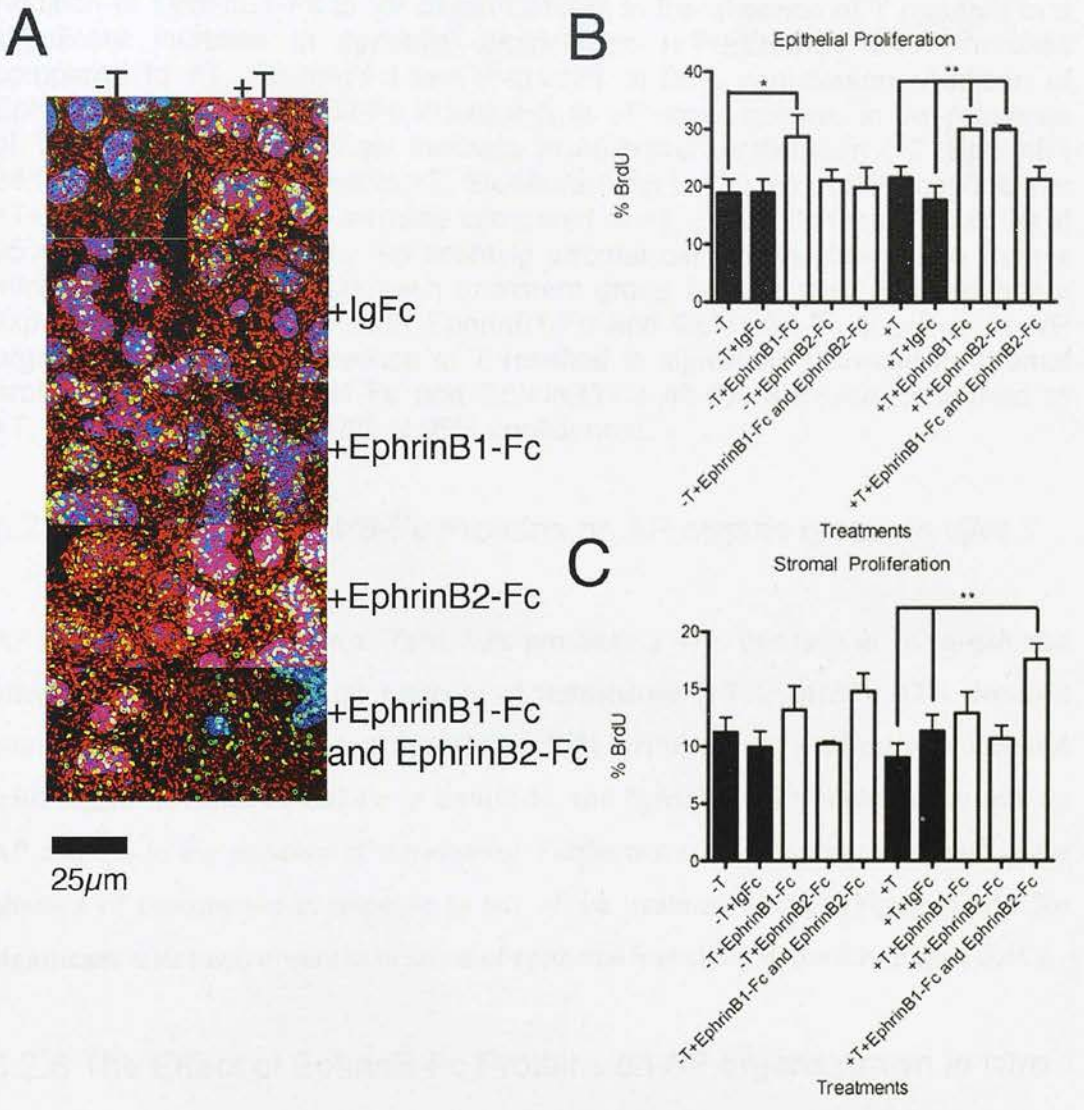

Figure 5.4 The Effect of EphrinB-Fc Proteins on proliferation of epithelial and stromal cells in the distal region of VP organs cultured for 6 days. Day 0 rat VP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M) and/or Fc control, EphrinB1-Fc, EphrinB2-Fc, or EphrinB1-Fc and EphrinB2-Fc (4μg/ml). All images taken at X40 magnification. Asteriks on graphs indicate statistical significance (* = $P<0.05$; **=$P<0.01$) **A**. BrdU incorporation in distal regions to the urethra was detected by IHC (green, yellow when co-localised to nuclei) and epithelial cells were distinguished by IHC for pan-Cytokeratin (blue). Nuclei were counterstained with propidium iodide (red). **B**. Data representing epithelial cell proliferation taken from a minimum of 2 organs from each treatment group from at least 3 independent experiments.

Addition of EphrinB1-Fc to VP organ cultures in the absence of T resulted in a significant increase in epithelial proliferation (+T+EphrinB1 34% increase compared to +T, Student's t test P=0.0141 at 95% confidence). Addition of EphrinB1-Fc and EphrinB2-Fc individually to VP organ cultures in the presence of T resulted in a significant increase in epithelial proliferation (+T+EphrinB1 28.06% increase compared to +T, Student's t test P=0.0191 at 95% confidence; +T+EphrinB2-Fc 28.04% increase compared to +T, Student's t test P=0.0109 at 95% confidence). **C**. Data representing stromal cell proliferation taken from a minimum of 2 organs from each treatment group from at least 3 independent experiments. Addition of both EphrinB1-Fc and EphrinB2-Fc together to VP organ cultures in the presence of T resulted in significant increase in stromal proliferation (+T+EphrinB1-Fc and EphrinB2-Fc 48.6% increase compared to +T, Students t test P=0.0009 at 95% confidence).

5.2.5 The Effect of EphB-Fc Proteins on AP organs grown *in vitro*

AP organ cultures treated with EphB3-Fc produced a 47% decrease in AP organ size after 6 days of culture in the presence of testosterone (+T+EphB3-Fc 47% decrease compared to +T, Students t test P=0.0027 at 95% confidence). No effect was observed with regard to either EphB2-Fc or EphB2-Fc and EphB3-Fc compound treatments on AP cultures in the presence of testosterone. Furthermore, no effect was observed in the absence of testosterone in response to any of the treatment groups (Figure 5.5B). No significant effect was observed in terms of epithelial branching in the AP (Figure 5.5C).

5.2.6 The Effect of EphrinB-Fc Proteins on AP organs grown *in vitro*

The addition of EphrinB-Fc ligand proteins did not have a significant effect on AP organ size or epithelial branching, in either the presence or absence of testosterone (Figures 5.6B and C).

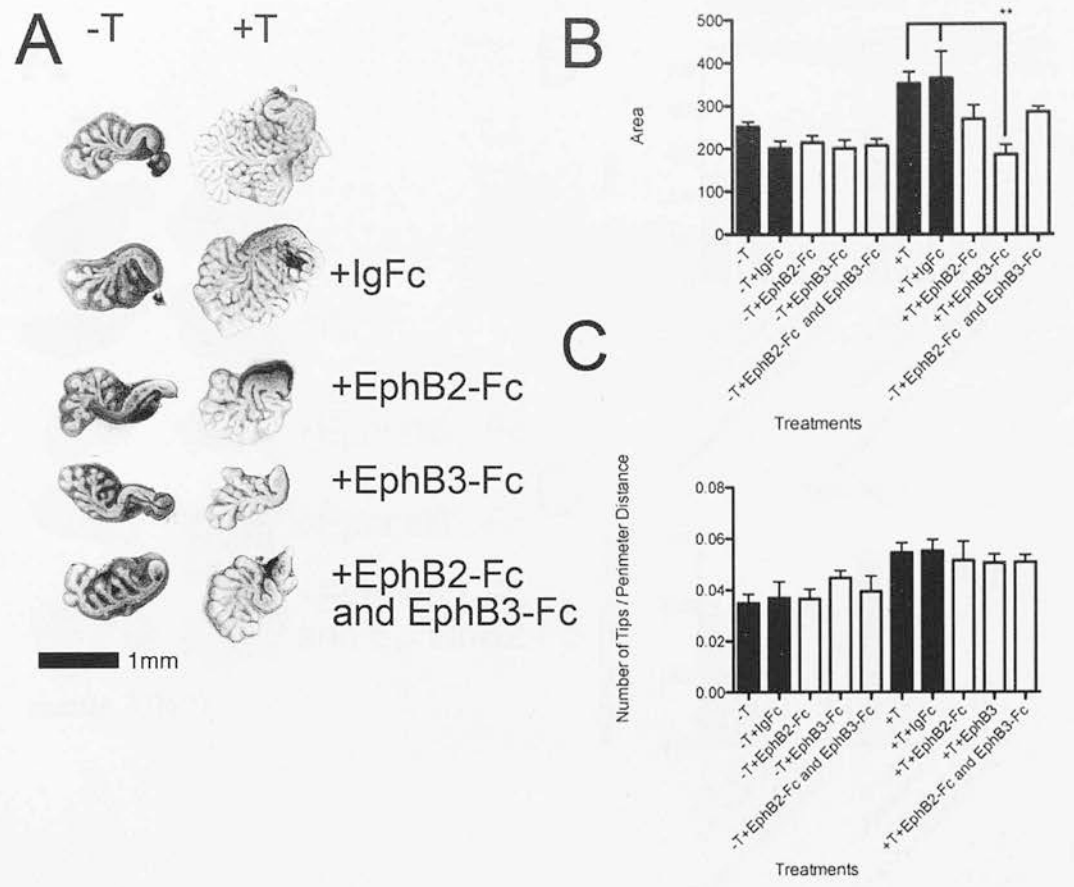

Figure 5.5. The Effect of EphB-Fc Proteins on AP organ size and epithelial branching. Day 0 rat AP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M)) and/or Fc control, EphB2-Fc, EphB3-Fc, or EphB2-Fc and EphB3-Fc (4µg/ml). Organs were imaged under 4X magnification using light microscopy before fixation. Asteriks on graphs indicate statistical significance (* = $P<0.05$). The data represents a minimum of 2 organs from each treatment group from at least 3 independent experiments. **A.** Representative pictures of AP organ cultures. **B**. The 2D AP organ area was measured using Image J freeware from NIH. Area units were measured in pixels. Addition of EphB3-Fc to AP organ cultures in presence of T resulted in a significant decrease in area (+T+EphB3-Fc 47% decrease compared to +T, Students t test $P=0.0027$ at 95% confidence). **C**. The number of epithelial tips around the distal perimeter from each treatment were counted and displayed as the ratio of tip number and perimeter distance.

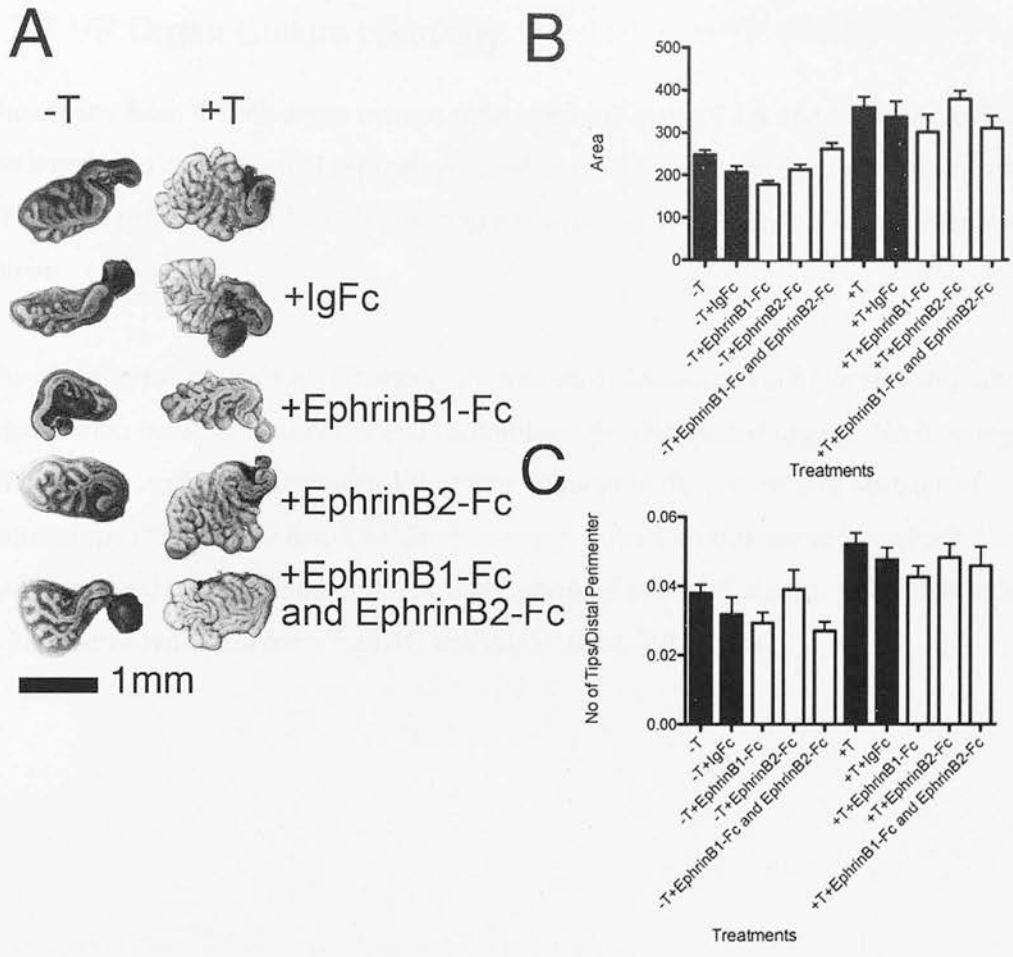

Figure 5.6. The effect of EphrinB-Fc Proteins on AP organ size and epithelial branching. Day 0 rat AP organs were cultured for 6 days in the presence and absence of testosterone (10^{-8}M) and/or Fc control, EphrinB1-Fc, EphrinB2-Fc, or EphrinB1-Fc and EphrinB2-Fc (4µg/ml). Organs were imaged under 4X magnification using light microscopy before fixation. The data represents a minimum of 2 organs from each treatment group from at least 3 independent experiments. **A.** Representative pictures of AP organ cultures. **B.** The 2D AP organ area was measured using Image J freeware from NIH. Area units were measured in pixels. **C.** The number of epithelial tips around the distal perimeter from each treatment were counted and displayed as the ratio of tip number and perimeter distance.

5.2.7 VP Organ Culture Histology

The organs from the VP organ culture experiments (Figures 5.1A and 5.3A) were fixed, sectioned, and stained with haematoxylin and eosin (H+E). Images of H+E stains of the VP organs from the EphB-Fc (Figure 5.7) and EphrinB-Fc (Figure 5.8) experiments are shown.

The histological analysis of VP organs showed no differences in cellular morphology or organisation between the control and recombinant protein treated organs. No histological difference was observed between VP organs cultured in the presence or absence of testosterone (Figures 5.7 and 5.8). The histology showed no difference in epithelial bud size between the control and EphB-Fc and EphrinB-Fc treated organs. This observation is in contrast with data from the IHC analysis of these VP organs.

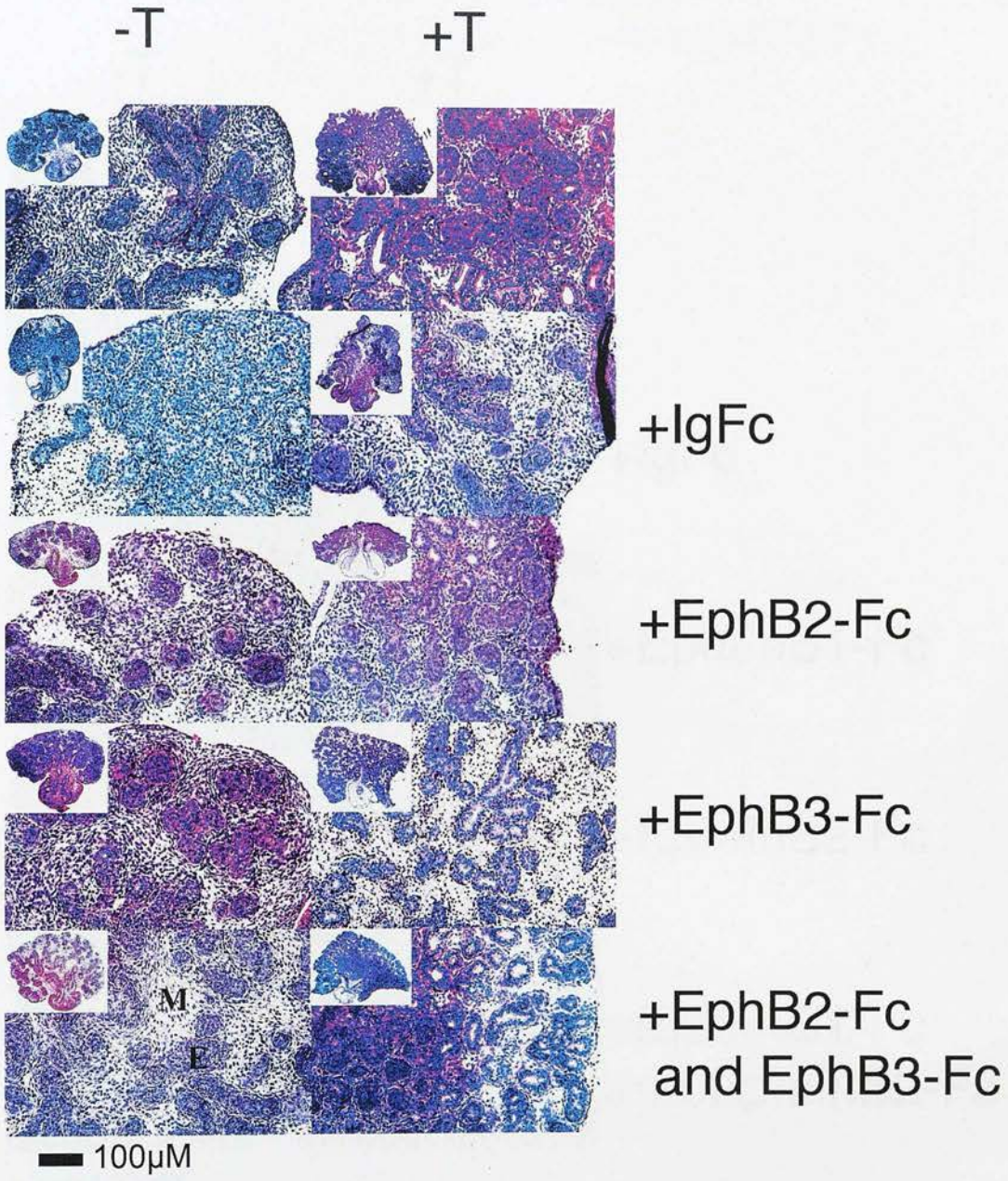

Figure 5.7 Histology of the EphB-Fc treated VP organ cultures. The organs from the VP organ culture were fixed, sectioned, and stained with haematoxylin and eosin. This was a repeated at least 3 times using the slides from 3 or more different experimental repeats. The scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

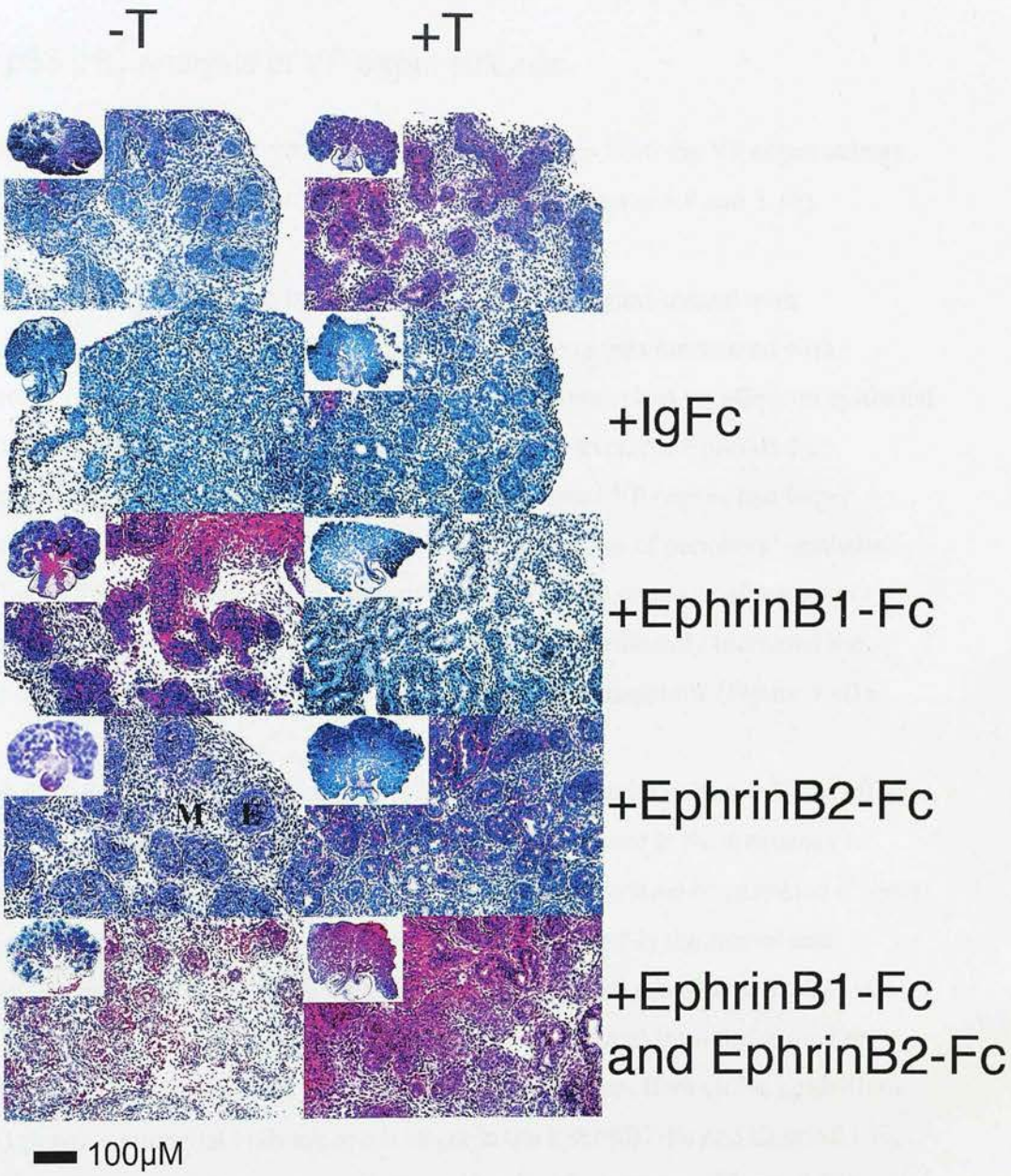

Figure 5.8 Histology of the EphrinB-Fc treated VP organ cultures. The organs from the VP organ culture were fixed, sectioned, and stained with haematoxylin and eosin. This was repeated at least 3 times using the slides from 3 or more different experimental repeats. The scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

5.2.8 p63 IHC analysis of VP organ cultures

Histological sections from the control and treatment groups from the VP organ culture experiments underwent IHC using an anti-p63 antibody (Figures 5.9 and 5.10).

The protein p63 is expressed in basal epithelial cells. The organs treated with testosterone produced more epithelial branching than the organs not treated with testosterone (Figures 5.9 and 5.10). The EphB-Fc experiments had no effect on epithelial organisation within the developing VP (Figure 5.9). However, the EphrinB-Fc experiments had a biological effect. The EphrinB-Fc treated VP organs had larger peripheral epithelial buds (Figure 5.3A) but a reduced number of peripheral epithelial buds (Figure 5.3C) compared to the control VP organs in the presence of testosterone. Additionally, EphrinB1 and EphrinB2 ligand treatments significantly increased the number of proliferating epithelial cells in the presence of testosterone (Figure 5.4B).

The EphrinB-Fc proteins had observable effects on the VP organ cultures. These effects were an increase in epithelial bud size (Figure 5.3A), a decrease in the frequency of epithelial buds (Figure 5.3C), and an increase both the proliferation of epithelial (Figure 5.4B) and stromal (Figure 5.4C) cells. EphrinB1 is expressed in the stroma and EphrinB2 is expressed in the epithelium. From these data, two possible rationales from for the observed effects are possible. Either the EphrinB1 signal from the stroma or EphrinB2 signalling from within the epithelium has a positive effect on the epithelium. The p63 positive epithelial buds are much larger in the EphrinB2-Fc and EphrinB1-Fc and EphrinB2-Fc treated VP organs as indicated by the black arrows (Figure 5.10).

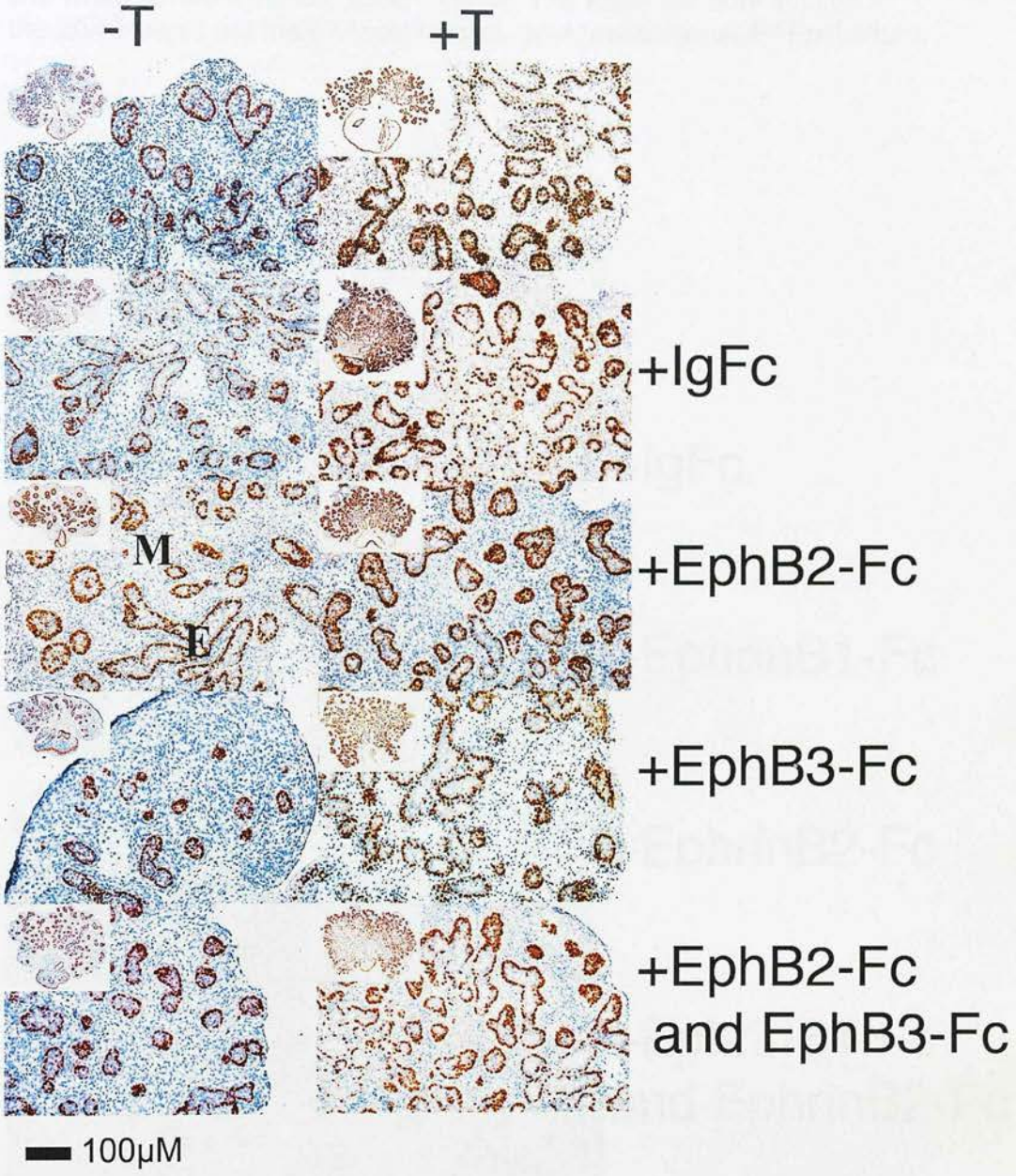

Figure 5.9 p63 IHC analysis of the EphB-Fc Proteins on VP organ cultures. The organs taken from the repeats of the experiment shown in Figure 5.1A were fixed and sectioned onto slides and examined using p63 IHC. The brown stained areas are the basal cells of the branching epithelial buds. This was a repeated at least 3 times using the slides from

3 or more different experimental repeats. The scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

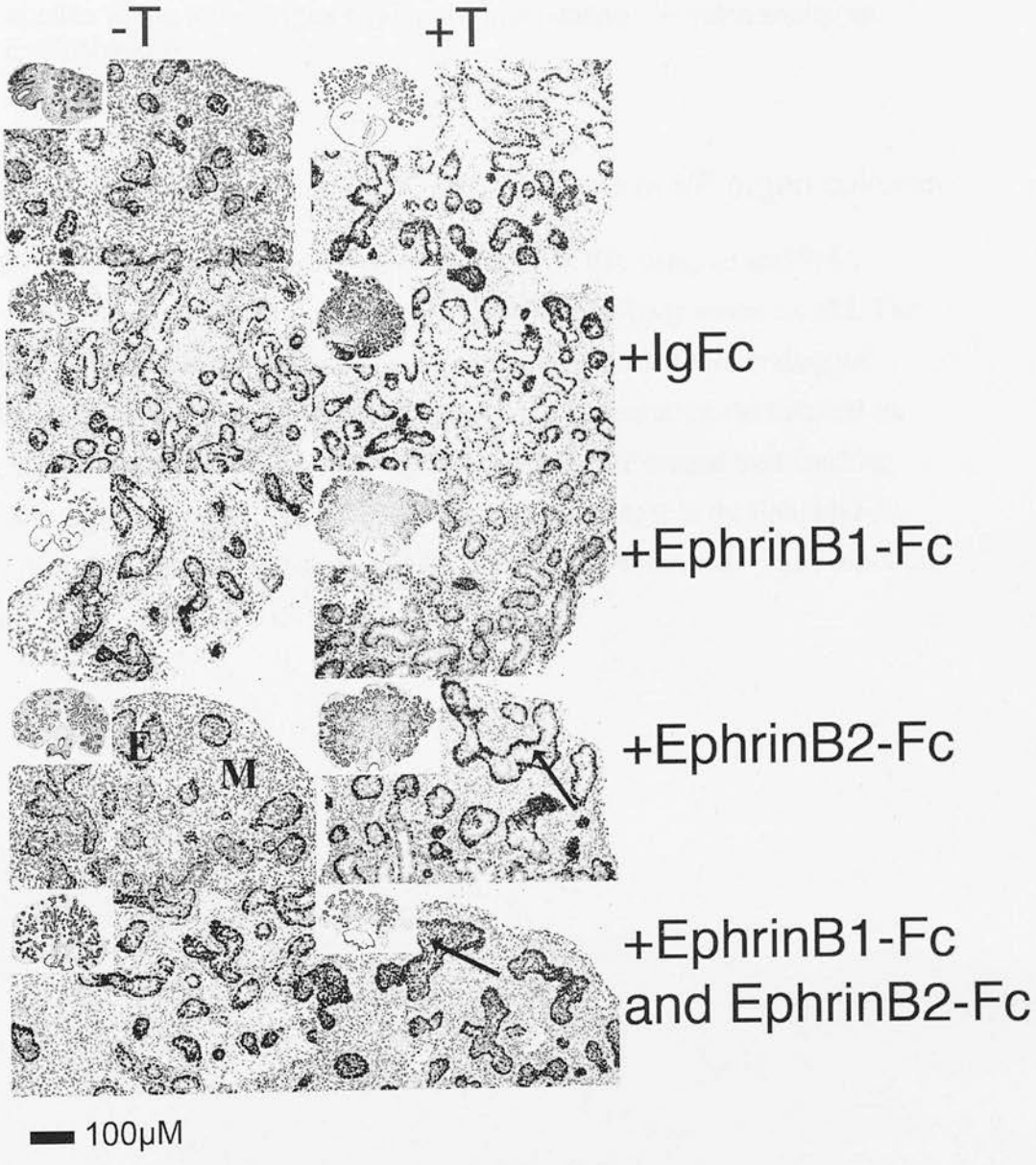

Figure 5.10 p63 IHC analysis of the EphrinB-Fc Proteins on VP organ cultures. The organs taken from the repeats of the experiment shown in Figure 5.3A were fixed and sectioned onto slides and examined using p63 IHC. The brown stained areas are the basal cells of the branching

epithelial buds. The epithelial buds in the EphrinB2-Fc and both EphrinB1 and EphrinB2-Fc treated VP organs are larger than the controls in the presence of testosterone. This was a repeated at least 3 times using the slides from 3 or more different experimental repeats. The scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

5.2.9 Smooth Muscle Actin (SMA) IHC Analysis of VP organ cultures

Slides from the VP organ culture experiments underwent IHC using an anti-SMA primary antibody (Figures 5.11 and 5.12). The anti-SMA antibody marks the SM. The EphB-Fc experiments had no effect on the organisation of the SM surrounding the epithelial buds within the developing VP (Figure 5.11). Furthermore, the EphrinB-Fc treatments had no effect on the stromal organisation of the VP around the branching epithelial buds (Figure 5.12). The epithelial buds appeared larger in the EphrinB2-Fc and EphrinB1-Fc and EphrinB2-Fc treated VP organs when compared to the controls, as indicated by the black arrows (Figure 5.12).

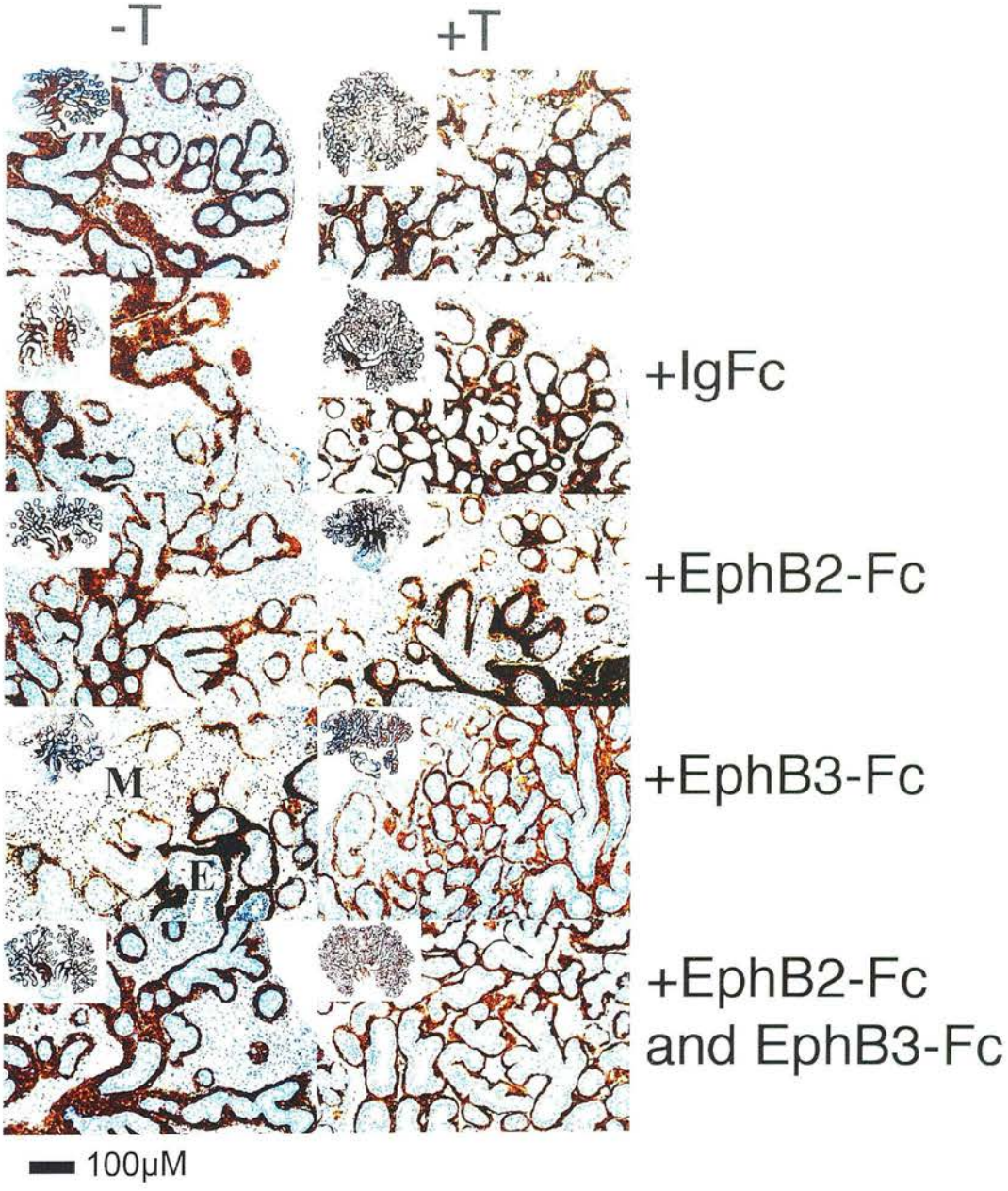

Figure 5.11 SMA IHC analysis of the EphB-Fc Proteins on VP organ cultures. The organs taken from the repeats of the experiment shown in Figure 5.1A were fixed and sectioned onto slides and examined using SMA IHC. The brown stained areas are the areas of smooth muscle surrounding the branching epithelial buds. This was a repeated at least 3 times using the slides from 3 or more different experimental repeats. The

scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

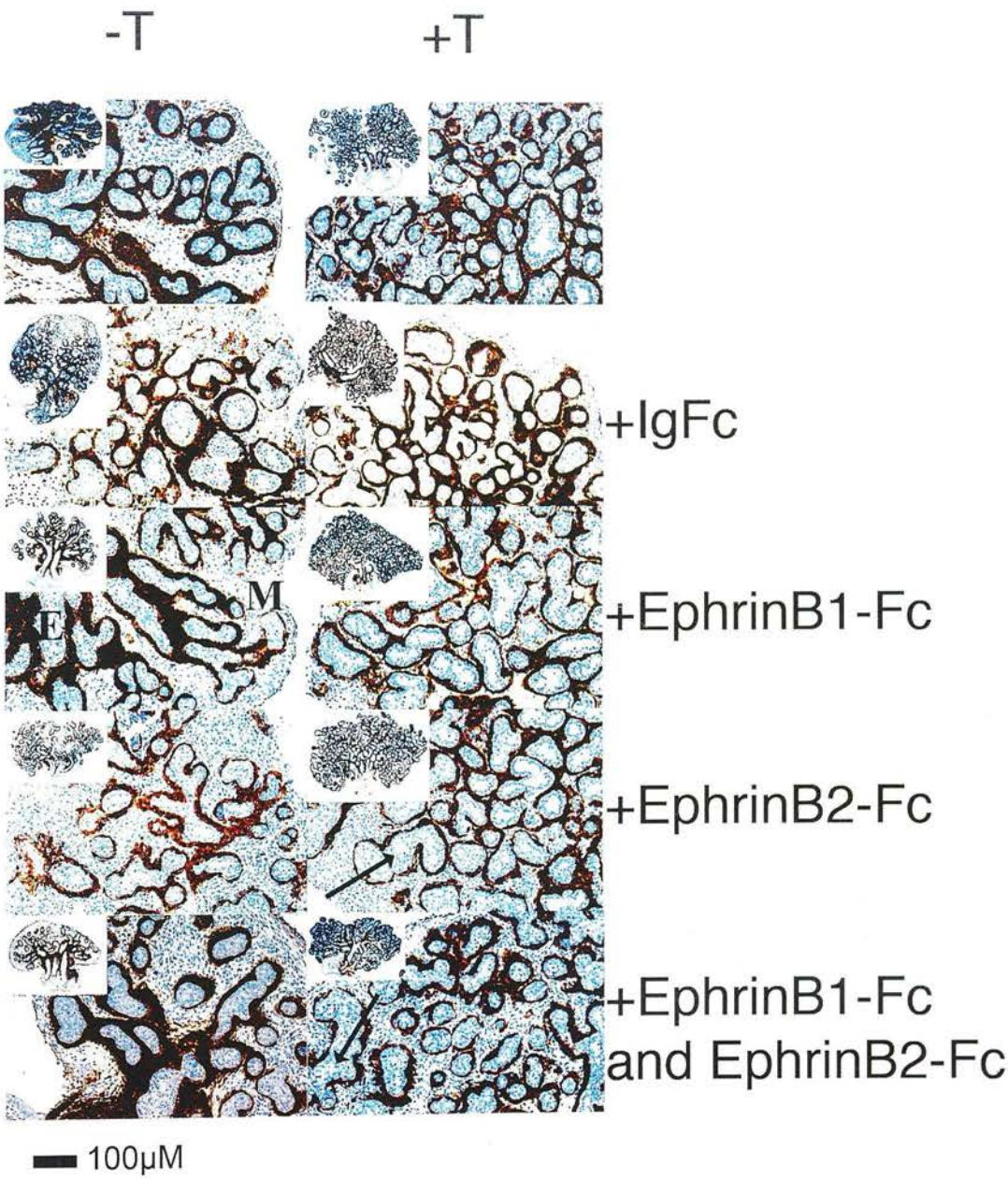

Figure 5.12 SMA IHC analysis of the EphrinB-Fc Proteins on VP organ cultures. The organs taken from the repeats of the experiment shown in Figure 5.3A were fixed and sectioned onto slides and examined using SMA IHC. The brown stained areas are the areas of smooth

muscle surrounding the branching epithelial buds. This was a repeated at least 3 times using the slides from 3 or more different experimental repeats. The scale bar only applies to the 20X images not the 4X inset images. M=Mesenchyme, E=Epithelium.

5.3 Discussion

We suggest EphrinB-EphB signalling has a role in VP development and may have a role in AP development. The addition of EphB2-Fc and EphB3-Fc to VP organs inhibited VP development (Figure 5.1). Both EphB2-Fc and EphB3-Fc treatments reduced VP organ size and the number of ductal tips in the presence of testosterone, though only EphB2-Fc had a significant effect with regard to the epithelial branching (Figure 5.1). When EphB2-Fc and EphB3-Fc were added together to VP organs no effect was observed on either area or epithelial branching. It could be expected that a compound effect would be seen resulting in a greater decrease in area as both EphB2-Fc and EphB3-Fc might function additively. However, the presence of both EphB2-Fc and EphB3-Fc in culture had no effect on VP organ size and the reason for this is not clear.

EphB-Fc addition had little effect upon VP cellular proliferation although EphB2-Fc did increase stromal proliferation by 35% in the absence of testosterone (Figure 5.2C). The EphB2-Fc and EphB3-Fc proteins were not membrane bound. The addition of the EphB2-Fc and EphB3-Fc proteins probably inhibited EphB signalling because most of the endogenous EphrinB ligands were likely bound by the EphB-Fc proteins. Furthermore, addition of the EphB2-Fc and EphB3-Fc proteins probably stimulated EphrinB reverse signalling as all endogenous EphrinB ligands were likely bound to an EphB-Fc protein. Decreased organ size, decreased epithelial branching and probable increased cell proliferation were the changes observed from the addition of the EphB-Fc proteins to VP organ cultures. The likely mechanisms which caused these observed changes was a decrease in EphB forward signalling, an increase in EphrinB reverse signalling or a combination of the two. Additionally, EphrinB1 is expressed in the

stroma and EphrinB2 is expressed in the epithelium. Therefore it is likely that both the epithelia and the stroma are affected as both express different EphrinB ligands.

The reduced organ size and level of epithelial branching and the trend increase in the number of proliferating stromal and epithelial cells appear to contradict each other. This may suggest that another factor, not the number of proliferating cells, is responsible for the decrease in organ size and epithelial branching of the EphB-Fc treated VPs. Additionally, only the cells at the distal perimeter of the VP organs was measured, ignoring the proximal cells. The increase in the number of proliferating epithelial and stromal cells not being statistically significant in the presence of testosterone is important (Figures 5.2B and C). Given that the organ size and degree of epithelial branching decrease after EphB2-Fc and EphB3-Fc treatment, it is likely that the number of proliferating epithelial and stromal cells would be less. However, a trend increase in both numbers of proliferating epithelial and stromal cells was observed. This increase was not significant; with the exception of the effect of EphB2-Fc upon the number of proliferating VP stromal cells, in the absence of testosterone (Figure 5.2C). Therefore, apart from this exception, the cellular proliferation data only partially contradicts the decrease in VP organ size and degree of epithelial branching as no overall statistically significant increase in the number of proliferating VP cells was observed. Another possible explanation for observed trends in VP organ size, epithelial branching and cellular proliferation would be smaller individual cells. However, there is no evidence for this in the histology or the IHC data.

The addition of EphrinB ligands to VP organ culture increased organ size, reduced epithelial branching and increased cell proliferation. EphrinB1-Fc and EphrinB2-Fc treatments increased VP organ size (Figure 5.3B) and decreased VP epithelial branching (Figure 5.3C). The EphrinB1-Fc and EphrinB2-Fc treatments increased the level of VP epithelial cell proliferation (Figure 5.4B). The increase in the number of proliferating epithelial cells reinforces the concept that EphrinB1 (expressed in the stroma) mediates a paracrine signal from the stroma to the epithelium and that EphrinB2 (expressed in the

epithelia) mediates a juxtacrine signal within the epithelium. These ligands may act together as positive regulators of VP development. The increase in the number of proliferating epithelial cells may account for the increased size of the epithelial buds in the EphrinB-Fc treated organs (Figure 5.3A). The individual EphrinB ligand treatments produced a statistically significant increase in the number of VP epithelial cells but the combined EphrinB ligand treatment did not. The individual EphrinB ligand treatments produced no effect on the number of proliferating VP stromal cells whereas the combined treatments gave a statistically significant increase in the number of proliferating VP stromal cells. Taken together, these results suggest VP epithelial proliferation is increased via EphB2 forward signalling in response to EphrinB1 or EphrinB2 stimulation. Furthermore, increased VP stromal cell proliferation appears to result from combined EphrinB1 and EphrinB2 stimulation of EphB3 forward signalling.

The epithelial buds of the EphrinB-Fc treated VP organs appear larger than the epithelial buds of control VP organs (Figure 5.3A) Furthermore, the p63 IHC of the EphrinB-Fc addition treatments (Figure 5.10) and the SMA IHC of the EphrinB-Fc addition treatments (Figure 5.12) also showed enlarged epithelial buds. These enlarged epithelial buds could explain the decreased degree of epithelial branching in response to EphrinB1-Fc, EphrinB2-Fc, and addition of both treatments. If there were larger individual buds, then there would be less space for epithelial branching in the stroma, assuming there was no change in stromal area. However, these observations were not quantified. EphrinB1 paracrine signalling from the stroma to the epithelium or EphrinB2 juxtacrine/autocrine signalling from within the epithelial itself are the two likely mechanisms to regulate in the increased epithelial bud size. EphrinB1-Fc and EphrinB2-Fc addition would affect the levels of EphB signalling in the VP in two ways. Either by binding to the EphB3 receptor expressed in the stroma or the EphB2 receptor expressed in the epithelia. Altered levels of EphB2 signalling in the epithelium could be directly responsible for the enlarged epithelial buds or an indirect effect from increased levels of EphB3 signalling in the stroma. Alternatively, both signalling mechanisms could be involved and each is partially responsible for the enlargement of the epithelial buds.

The recombinant EphrinB1-Fc and EphrinB2-Fc proteins, added exogenously, probably stimulated EphB forward signalling because most of the endogenous EphB receptors were likely bound by the EphrinB-Fc proteins. Furthermore, addition of the EphrinB1-Fc and EphrinB2-Fc proteins probably inhibited EphrinB reverse signalling as all endogenous EphB receptors were likely bound to an EphrinB-Fc protein. Increased organ size, decreased epithelial branching and probable increased cell proliferation were the changes observed from the addition of the EphrinB-Fc proteins to VP organ cultures. The likely mechanisms which caused these observed changes was an increase in EphB forward signalling, a decrease in EphrinB reverse signalling or a combination of the two. Additionally, EphB2 is expressed in the epithelia and EphB3 is expressed in the stroma. Therefore it is likely that both the epithelia and the stroma are affected as both express different EphB receptors.

The AP organ culture system provided a second primary organ *in vitro* model to elucidate the functions of particular proteins. A limitation of the AP culture system was the technical difficulty of obtaining histological sections on a microtome due to the small size of the organs.

AP organ size decreased by 47% in response to EphB3-Fc in the presence of testosterone (Figure 5.5B). This suggests EphB-EphrinB signalling role has a role in AP development mediated via the EphB3 receptor. The decrease in AP organ size in response to EphB3-Fc is consistent with the reduction in VP organ size in response to EphB3-Fc treatment (Figure 5.1B). This suggests that EphB3 has a role in the growth of both VP and AP.

None of the EphrinB-Fc ligand treatments produced an effect in the AP organ culture, as these ligand treatments did in the VP organ culture. If an EphB receptor has a critical role, then one of its ligands is likely to also have a role in AP development. Another Ephrin ligand, not EphrinB1 or EphrinB2, may be responsible. EphB receptors can

sometimes bind to the EphrinA ligands. For example, it is well-known receptor EphA4 and ligand EphrinA5 can interact with EphrinB2 and EphB2, respectively (Bouzioukh et al., 2007; Himanen et al., 2004) The EphA-EphrinA family were not examined in this study.

Histological and IHC analysis were used to determine if the EphB-Fc and EphrinB-Fc recombinant protein experiments had an effect on the VP cellular morphology and organisation. The structure of the developing VPs was examined using H+E staining followed by the epithelial and stroma being investigated individually using epithelial and stromal markers. From the H+E analysis (Figures 5.7 and 5.8) no effect on the cellular morphology and organisation of the VP was observed. Additionally, the EphB-Fc treatments appeared to have no effect on VP cellular morphology and organisation (Figures 5.9 and 5.11). However, in response to EphrinB-Fc ligand treatment there appeared to be an increase in the size of individual buds as shown by p63 and SMA immunohistochemical analysis in Figures 5.10 and 5.12.

The EphB receptors and EphrinB ligands are best characterised as chemotactic guidance cues in neuronal development (Hinck, 2004) and as signalling molecules in organogenesis (Merlos-Suarez and Batlle., 2008). The experiments described here show that EphB and EphrinB proteins have a role in prostate organogenesis. The role of EphB-EphrinB signalling in the prostate has not been researched as thoroughly as other growth factors such as Fibroblast Growth Factor 10 (FGF10) and Transforming Growth Factor β (TGFβ). FGF10 is a known mesenchyme paracrine regulator of epithelial growth in the prostate and seminal vesicle (Thomson and Cunha, 1999), and TGFβ has an effect on the growth of the rat prostate epithelium (Tomlinson et al., 2004). These are examples of potent secreted growth factors that affect cell positioning and organisation. The members of the EphB signalling system are not secreted but act as extracellular signals in cell-cell interactions and have a role in rat prostate development.

As an overview, the addition of EphB-Fc receptor proteins to VP cultures decreased the organ size (Figure 5.1A and B) whereas the addition of EphrinB-Fc ligand proteins increased the organ size (Figure 5.3A and B). The number of VP peripheral epithelial tips decreased in response to both EphB-Fc receptor proteins (Figure 5.1C) and EphrinB-Fc ligand proteins (Figure 5.3C).

Furthermore, the addition of both types of protein increased the overall cellular proliferation rates of VP epithelial and stromal cells (Figures 5.2 and 5.4). The EphrinB-Fc ligand proteins appeared to increase the size of the VP epithelial buds (Figures 5.3A, 5.10, and 5.12). The addition of EphB-Fc receptor proteins to AP cultures decreased the organ size (Figure 5.5A and B).

Discussion

6.1 The Identification of transcripts expressed in prostate mesenchyme

A whole mount in situ hybridisation (WISH) analysis was conducted to identify transcripts with expression patterns restricted to prostate mesenchyme. The candidates came from a SAGE transcript profile of the VMP (Vanpoucke et al., 2007). For a transcript to be included in the WISH analysis; it required both a putative NCBI Nucleotide sequence file and the preparation of a good quality RNA probe. 194 transcripts were selected for the WISH analysis and were divided into three groups of transcripts. These were the statistically selected group, the non-statistically selected group and the intuitive transcript group. The statistically selected group encompassed the transcripts from the SAGE data that had a VMP to VSU ratio of 5:0. This ratio was the minimum for a statistically significant difference between the VMP tag count and the VSU tag count. In contrast, and as a control to this statistically selected group, the non-statistically selected group encompassed the transcripts with a VMP to VSU ratio of 4:0. As this ratio was less than 5:0, it was not statistically significant, but could be suggested to show a trend towards VMP enrichment. In addition to these two groups, the intuitive transcript group consisted of genes that expressed either membrane or secretory proteins with a statistically significant VMP to VSU ratio (i.e. greater than 5:0). The criteria described above removed 164 of the 193 transcripts from the WISH analysis. From the remaining 30 transcripts that were analysed, 13 transcripts were identified as mesenchyme enriched.

There were 54 transcripts in the statistically selected group (Table 3.1). WISH analysis of the statistically selected group was expected to determine what proportion of these mRNAs showed VMP/mesenchymal expression. We hoped to define the exact number of mesenchyme expressed mRNAs in this group, to determine how efficient the SAGE and

statistical analysis was. From the 54 transcripts, 19 transcripts did not have a NCBI Nucleotide sequence file. Of the remaining 35 transcripts, 10 transcripts were randomly selected for WISH analysis. Of these 10 transcripts, 4 did not a have a good quality RNA probe. From the remaining 6 transcripts, 2 transcripts were identified as stromal. The two stromal transcripts discovered were the Ras GTPase, RRAD/RasD (Caldwell et al, 1996) and tyrosine kinase receptor, EphB3 (Bohme et al., 1993) (Figure 3.3).

The non-statistically selected group of transcripts provided randomly selected controls for finding mRNAs in the mesenchyme. However, the SAGE tag frequency of 4:0 (VMP:VSU) shows a trend towards VMP enrichment. In the non-statistically selected group, there were 122 transcripts (Table 3.2). 94 transcripts did not have a NCBI Nucleotide sequence file leaving 28 transcripts to be analysed by WISH. Of these 28 transcripts, 16 did not a have a good quality RNA probe. From the remaining 12 transcripts, 4 transcripts were identified as stromal. These were ETL (Nechiporuk et al, 2001), CRIP2 (Karim et al., 1996), LAT3 (Babu et al., 2003), and RBMS1 (Negishi et al., 1994) (Figures 3.4 and 3.5).

The intuitive transcript group consisted of a subset of the 219 list and the statistically selected group contained mRNAs that encoded membrane bound and secreted proteins. Transcripts that encode such proteins make for interesting paracrine factor candidates. There were 17 transcripts in total in the intuitive transcript group (Table 3.3). 2 transcripts did not have a NCBI Nucleotide sequence file leaving 15 transcripts to be analysed by WISH. Of these 15 transcripts, 3 did not a have a good quality RNA probe. From the remaining 12 transcripts, 7 transcripts were identified as stromal. These were Sema6D (Qu et al., 2002), SPARC (Mason et al., 1986), Decorin (Day et al., 1986), Tsukushi (Ohta et al., 2004), Sprouty1 (Gross et al., 2001), Sorcs2 (Rezgaoui et al., 2001) and Nell2 (Watanabe et al., 1996) (Figures 3.6 and 3.7).

In total, 13 transcripts were identified as expressed in the mesenchyme by WISH. The WISH analysis lacked a transcript with a RNA probe that could be used as a reliable epithelial control. Examining frozen cross-sections of the whole mounts exposed to potential epithelial

RNA probes may have provided more insight into why the RNA probes did not work. However, this was not done at the time the experiments were conducted. Furthermore, an epithelial RNA probe may have been obtained from a laboratory that had examined epithelial transcripts using WISH (Thomsen et al., 2008). The absence of an epithelial control RNA probe was a limitation of the WISH analysis since the epithelium could not be identified by this WISH analysis. However, this limitation did not invalidate the mesenchymal transcripts that were identified as a result of the WISH analysis.

It could be argued that the 13 transcripts that were identified by WISH as mesenchymal produced so non-specific binding as well. Due to the PCR products not being sequenced at the time of synthesis, it was impossible to determine if the hybridisation is 100% specific (Figures 3.2-3.7). However, as the PCR primers underwent homology checks and the PCR products were examined to ensure they were the correct size prior to cloning then the chances of the hybridisation not being specific was highly unlikely. Nevertheless, an additional method that would have ensured the hybridisation specificity was Northern blotting which would have identified the exact binding specificity of each RNA probe. Additionally, immunohistochemistry (IHC) could have been performed to examine the expression of the proteins that the 13 transcripts encode. This would have provided more information about the expression pattern of each WISH result. Being able to compare and contrast the expression patterns of both the RNA (WISH) and protein (IHC) would have been very insightful in the analysis of all 13 mRNAs.

4 out of 12 transcripts from the non-statistically selected group were identified as enriched in the mesenchyme compared to 2 out of 6 transcripts from the statistically selected group. Therefore the proportion of mesenchyme enriched transcripts in each group was similar, suggesting that statistics were not effective. However, this conclusion is not reliable as too few transcripts were examined in each group. A contributory factor to the small number of transcripts being analysed was the technical issues involved in the RNA probe production. If there were less technical issues for one particular group, that would have a bearing on the number of RNA probes available for the WISH analysis for that particular group. For

example, the statistically selected group experienced RNA probe production issues for 4 transcripts. Conversely, the non-statistically selected group experienced RNA probe production issues for 16 transcripts. These problems led to too few transcripts being analysed to determine if groups showed different proportions of mesenchyme enriched transcripts.

13 transcripts were identified as expressed in the mesenchyme. Few signalling pathways have previously been reported as restricted to the prostatic mesenchyme, although one example includes the Notch2/Dlk1 pathway (Orr et al., 2009). Two transcripts considered for further analysis, these were for the transmembrane receptors Sema6D and EphB3. Both of these receptors are members of large signalling pathway families. EphB3 and its family members are thoroughly characterised in more biological models in the scientific literature than Sema6D. Furthermore, the EphB family had more antibodies and recombinant proteins available. EphB3 was selected as the candidate transcript to further characterise in the developing rat prostate.

The tyrosine kinase receptor, EphB3, was chosen as a candidate for expression and functional analysis in rat prostate development because it is a signalling receptor that was enriched in the prostate stroma via WISH. The EphB family has 5 known receptors and 3 ligands in mammals. To establish if any other EphB family members were expressed in different rat urogenital tissues, PCR analysis was performed. The EphB2 and EphB3 receptors and the EphrinB1 and EphrinB2 ligands were strongly expressed in prostatic tissues. The EphB4 receptor and the EphrinB3 ligand were weakly expressed in the VP, VMP, and VSU tissues. However, these bands were less intense than the bands for the EphrinB1/B2 ligands and the EphB2/B3 receptors. Thus, EphrinB3 ligand and EphB4 receptor were not investigated further. It is possible EphrinB3 and EphB4 play a role in prostate development but this would require much further investigation. No EphB1 expression was reported (Figure 4.1). Due too their expression in the prostate (Figure 4.1), the EphrinB1 and EphrinB2 ligands and the EphB2 and B3 receptors were examined further.

6.2 EphB receptor and EphrinB ligand expression in the developing prostate

The EphB3 transcript was detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the mesenchyme. The EphB3 expression data in the male rat urogenital tracts suggested it was stromal. WISH showed EphB3 expressed in the mesenchyme of the VP and DLP, parts of the male d0 urogenital tract. In the female d0 urogenital tract, EphB3 mRNA was exclusively expressed in the VMP mesenchyme (Figure 4.2). The EphB3 mRNA and protein expression patterns were consistent with each other. The EphB3 protein was expressed in the mesenchyme but there was weak EphB3 mRNA expression in the urethral epithelium in the female (Figure 4.8). Consistent with these findings, EphB3 is expressed in the mesenchymal tissues during chicken development (Baker et al., 2001) and in murine palatal mesenchyme (Risley et al., 2008).

The temporal expression pattern of EphB3 mRNA was examined by quantitative real time PCR during VP development. Between e17 and postnatal d10, EphB3 receptor expression was approximately constant at 5 fold over TBP. The level of EphB3 expression increased to approximately 12.5 fold over TBP by d35 prior to the levels decreasing to approximately 1 fold over TBP by adulthood (Figure 4.4). The increase to 12.5 fold over TBP in EphB3 mRNA levels at d35 is unusual when compared to the expression patterns of the other EphB-EphrinB genes investigated in this study. The EphB2 receptor and EphrinB1/B2 ligand mRNA levels were approximately 1 fold over TBP at d35 postnatal (Figures 4.3, 4.5 and 4.6). It is possible that EphB3 may have a novel ligand at d35 that is not an EphrinB ligand. EphrinA ligands are known to interact with EphB receptors (Himanen et al., 2004) or perhaps a non-Ephrin ligand interacts with EphB3 at d35. The role of EphB3 signalling during VP development at d35 was not investigated. Alternatively, the high expression of EphB3 at d35 and the low expression of EphrinB1 at d35 may represent a repulsive juxtracrine mechanism within the stroma. Although there is no direct evidence for it, this

potential EphB3-EphrinB1 repulsion within the stroma may be androgen drive. It occurs at d35 which is when testosterone levels are very high in the bloodstream during puberty. More work would need to be conducted to see if EphB3 or EphrinB1 are androgen responsive. Collectively, the EphB3 expression data suggests that EphB3 is involved in either paracrine signalling in partnership with the epithelium or has a role in intra-stromal juxtacrine or autocrine signalling during rat prostate development.

The EphB2 protein was expressed in male UGT epithelium (Figure 4.9). Moreover, the EphB2 transcript was not detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the epithelium. These observations suggested that EphB2 expression was restricted to the epithelium. These observations were consistent with the epithelial specific expression of EphB2 in the developing ear epithelium (Cowan et al., 2000) and vascular endothelial cells (Salvucci et al., 2006). During VP development, the level of EphB2 mRNA (Figure 4.3) decreased from approximately 4 fold over TBP at e17 to approximately 1 fold over TBP by adulthood. This suggests any cell signalling through the EphB2 receptor, expressed predominantly in the epithelium, is pre-natal.

In genital tubercle development of the mouse, the expression pattern of EphB2 has been reported to be sexually dimorphic (Lorenzo et al., 2003). EphB2 is expressed is restricted to the male but is observed in female genital tubercles after exposure to dihydrotestosterone (DHT) in culture. This suggests that EphB2 is a candidate androgen-regulated gene in mouse genital development. EphB2 protein is predominantly epithelium in the male d0 UGT (Figure 4.9) but is also expressed in the stromal and epithelial compartments of the female d0 UGT (Figure 4.10). This suggests EphB2 has differential expression, regulated by the differences in the levels of circulating testosterone, therefore supporting EphB2 as a candidate androgen regulate gene in prostate development. Conversely, EphB2 was expressed in both male and female d0 UGTs (Figure 4.9 and 4.10) and there has been no difference in prostate size reported between wild type and EphB2 knockout mice (Yucel et al., 2007). These observations suggest EphB2 is not androgen regulated in the prostate and not essential to mouse prostate development. Furthermore, the addition of EphB2-Fc to VP

organs resulted in a significant increase in the percentage of proliferating stromal cells in the absence of testosterone (Figure 5.3C). The addition of EphB2-Fc to VP organs was the only treatment that had a positive effect on the percentage of proliferating VP stromal cells in the absence of testosterone. This observation suggests EphB2 does not require testosterone to be present to elicit a biological effect. The possible androgen regulation of EphB2 in rat prostate development was not investigated.

EphrinB1 mRNA (Figure 4.2) and protein (Figures 4.11 and 4.12) expression was restricted to the mesenchyme. EphrinB1 was restricted to the peri-epithelial mesenchyme in the d0 male UGT. Moreover, the EphrinB1 transcript was detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the mesenchyme. EphrinB1 is expressed in the mesenchyme surrounding other developing organs such as the spinal cord (Jevince et al., 2006) and in the mesenchyme during developmental processes such as corneal angiogenesis (Kojima et al., 2007). Furthermore, EphrinB1 is restricted to the mesenchyme of terminal end buds during murine mammary branching morphogenesis, as shown by in-situ hybridisation (Kouros-Mehr and Werb., 2006). The EphrinB1 is clustered around the EphB2 positive epithelial buds and co-localises with EphB3 in the peri-epithelial mesenchyme, especially in the DLP and VP (Figure 4.11). This co-localisation reflects the fact that EphB-EphrinB interactions are highly clustered and dependent on close cell-to-cell proximity for the EphB mediated signal to be transmitted (Davis et al., 1994). EphrinB1 is a ligand for both the EphB2 and EphB3 receptors. EphB3 was expressed in the mesenchyme and EphB2 in the epithelium during rat prostate development. Taken together, this data suggests EphrinB1-EphB signalling occurs in a paracrine fashion (via EphB2) to the epithelium and/or a juxtacrine/autocrine fashion (via EphB3) within the mesenchyme.

EphrinB1 expression during VP development was similar to EphB2. At e17, the EphrinB1 mRNA level at e17 was approximately 7 fold over TBP that decreased to 2 fold over TBP. At e17, the EphB2 mRNA expression level was approximately 4 fold over TBP and decreased to approximately 1 fold over TBP by adulthood. The EphrinB1 ligand was expressed in the stroma and the EphB2 receptor in the epithelium. This expression data

taken together suggests that EphrinB1 and EphB2 interact in a paracrine fashion as a mechanism of stromal-epithelial interaction during rat VP development.

EphrinB2 protein was expressed in the epithelium of the male d0 UGT (Figure 4.13). In the female UGT, EphrinB2 was predominantly expressed in the SM layer with a degree of expression in the urethral epithelium and the VMP (Figure 4.14). The EphrinB2 transcript was not detected by the SAGE analysis (Table 4.1), suggesting that it was expressed in the epithelium. Furthermore, EphrinB2 expression was restricted to the epithelium in other tissues including the intestine (Hafner et al., 2005) and pulmonary vasculature (Schwarz et al., 2009). However, EphrinB2 was expressed in different tissue types in the development of other biological systems. During angiogenesis in the mouse, EphrinB2 was expressed in the epithelium and mesenchyme (Adams et al., 1999; Korff et al., 2006). Collectively, this data suggests that EphB2-EphrinB2 juxtacrine/autocrine signalling is active within the epithelium. Moreover, it is possible than EphrinB2 associates with the mesenchymal EphB3, providing a paracrine signalling mechanism between from epithelium to the mesenchyme.

EphrinB2 was expressed in the epithelium and EphB3 in the mesenchyme in the male rat d0 UGT. This is not the only difference between this ligand and receptor in terms of expression. The expression of EphrinB2 mRNA during VP development greatly decreased between e17 and maturity (Figure 4.6), therefore EphrinB2 is unlikely to be the ligand that may interact with the EphB3 receptor at d35 (Figure 4.4.). There was no increase at d35 in EphrinB2 expression like EphB3 during VP development. Therefore, it is likely that any signalling involving EphrinB2 in VP development is active at either prenatal or early postnatal time points.

6.3 The function of EphB receptors and EphrinB ligands in the developing prostate

VP development was inhibited in response to EphB2-Fc and EphB3-Fc proteins. VP organ size was significantly reduced by both EphB2-Fc and EphB3-Fc treatments in the presence of testosterone. EphB2-Fc decreased the epithelial branching morphogenesis significantly after six days of *in vitro* culture (Figure 5.1). These observations suggest EphB2 signalling has a function in rat VP development. No effect on VP organ size or epithelial branching morphogenesis was observed when EphB2-Fc and EphB3-Fc were added to the VP cultures together. A synergistic effect may have been expected, resulting in a greater reduction in area as both EphB2-Fc and EphB3-Fc decrease VP organ size individually. The two EphB-Fc proteins may interfere with each others binding the endogenous EphrinB ligands in some way. It could be suggested that antibodies, raised against the appropriate EphB receptors and EphrinB ligands, could be used instead of recombinant EphB-Fc and EphrinB-Fc to determine the functionality of EphB signalling in prostate development. The organ culture experiments performed as part of this thesis used recombinant proteins for two reasons. Firstly, using recombinant protein is the conventional experimental practice when investigating the role of EphB-EphrinB signalling in vitro culture assays (Kayser et al., 2006; Alfaro et al., 2007; Kojimo et al., 2007). Secondly, an antibody would not elicit the same biological effect as a recombinant protein in terms of downstream intracellular signalling because the binding affinity would not be as high as a recombinant protein. This would be a feature of the exogenous reagent that would be desirable in a functional experiment. For this reasons, EphB and EphrinB recombinant proteins were used in these organ culture experiments as opposed to antibodies. The negligible differences in the molecular weights of the four exogenous recombinant proteins probably had little effect on the experimental outcome.

EphB2-Fc and EphB3-Fc gave a trend increase in the percentage of proliferating VP epithelial and stromal cells in the presence of testosterone (Figure 5.2B). EphB2-Fc and

EphB3-Fc may bind to endogenous EphrinB1 in the stroma, and EphrinB2 in the epithelium, to increase in the percentage of proliferating VP epithelial and stromal cells. This is similar to mesenchyme-epithelial EphB-EphrinB interactions during murine cardiovascular development (Adams et al., 1999). The EphB2-Fc and EphB3-Fc proteins can affect both EphrinB reverse signalling and EphB forward signalling. Therefore, the mechanisms by which the observed effects of decreased organ size, decreased epithelial branching morphogenesis and trend increase in the percentage of proliferating cells could be explained is a change in the level of EphrinB reverse signalling, EphB forward signalling or both.

The decreased organ size and level of epithelial branching, and the increase in epithelial and stromal proliferation were contrasting observations. The decrease in VP organ size and epithelial branching morphogenesis in the EphB2-Fc treated VP organs is not due to stromal and epithelial cell proliferation. The increase in the percentage of proliferating epithelial and stromal cells was not statistically significant in the presence of testosterone (Figures 5.2B and C). It would be likely that the percentage of proliferating cells would be less than the controls, given that the organ size and degree of epithelial branching morphogenesis decreased after EphB2-Fc and EphB3-Fc treatments. However, a trend increase in both the percentage of proliferating epithelial and stromal cells rates in the presence of testosterone was observed. Similarly contrasting observations have been reported before when recombinant TGFbeta1 was added to VP cultures (Tomlinson et al., 2004). The varying level of cellular differentiation may have been the cause of the contrasting observations. A decreased level of apoptosis at the periphery may of been another cause however this was not investigated. Peripheral cells are undifferentiated as opposed to the more differentiated cells at the centre of the VP. The percentage of proliferating VP cells was measured at the periphery of the organs where most of the cell proliferation was occurring. VP organ area includes the whole organ and the degree of epithelial branching is dependent the individual bud size not just the number of the epithelial buds. These differences may account for the contrasting observations of decreased VP organ size, decreased degree of epithelial branching and increased cellular proliferation. Decreased apoptosis

Individual EphrinB1-Fc and EphrinB2-Fc ligand treatments significantly increased VP organ size (Figure 5.3B), significantly reduced VP epithelial branching (Figure 5.3C), and significantly increased the percentage of VP proliferating epithelial cells (Figure 5.4B). Individual EphrinB1-Fc and EphrinB2-Fc treatments did not affect the percentage of VP proliferating stromal cells (Figure 5.4C). Combined EphrinB1-Fc and EphrinB2-Fc treatments did not affect VP organ size (Figure 5.3B) but did significantly decrease the degree of VP epithelial branching (Figure 5.3C). Combined EphrinB1-Fc and EphrinB2-Fc treatments did not affect the percentage of proliferating VP epithelial cells (Figure 5.4B), but did significantly increase the percentage of VP proliferating stromal cells (Figure 5.4C).

There was no synergistic effect on VP size or epithelial branching morphogenesis when EphB2-Fc and EphB3-Fc were added together (Figure 5.1). Similarly, there was no synergistic increase in either VP organ size (Figure 5.3B) or percentage of VP epithelial cells (Figure 5.4B) when EphrinB1-Fc and EphrinB2-Fc were added together. A synergistic effect may have been expected, resulting in a greater increase in VP area and percentage of proliferating VP epithelial cells since both EphrinB1-Fc and EphrinB2-Fc significantly increased VP organ size and percentage of proliferating VP epithelial cells individually. The two EphrinB-Fc proteins may interfere with each others binding the endogenous EphB receptors in some way.

EphrinB1-Fc and EphrinB2-Fc may bind to endogenous EphB2 in the epithelium and EphB3 in the stroma to increase in the percentage of proliferating VP epithelial and stromal cells. This is similar to mesenchyme-epithelial EphB-EphrinB interactions during murine cardiovascular development (Adams et al., 1999). The EphrinB1-Fc and EphrinB2-Fc proteins can affect EphB forward signalling and EphrinB reverse signalling. Therefore an alteration in the levels of these two signalling mechanisms may account for the observed effects of increased organ size, decreased epithelial branching and increase in the percentage of proliferating VP cells.

The distal epithelial buds of the EphrinB-Fc treated VP organs appeared larger in size than the control distal epithelial buds of the VP organs (Figure 5.3A). Moreover, the p63 (Figure 5.10) and the SMA (Figure 5.12) IHC of the EphrinB-Fc treated VP organs both show enlarged epithelial buds. A decreased degree of epithelial branching morphogenesis in response to EphrinB1-Fc, EphrinB2-Fc, and both treatments was observed (Figure 5.3C). The enlarged epithelial buds may explain the observations from Figure 5.3C. There would be a smaller degree of epithelial branching in the available stroma if there were larger individual buds.

The two probable mechanisms to cause the increased epithelial bud size are either EphrinB1 paracrine signalling from the stroma to the epithelium, or EphrinB2 juxtacrine/autocrine signalling in the epithelium. The level of EphB signalling in the VP could be affected by the EphrinB1-Fc and EphrinB2-Fc proteins binding to the EphB3 receptor expressed in the stroma or binding to the EphB2 receptor expressed in the epithelia. Changes in the level of EphB2 signalling in the epithelium may have caused the enlarged epithelial buds. Alternatively, an indirect effect from increased levels of EphB3 signalling in the stroma may have been responsible for the epithelial bud size increase via a paracrine stroma to epithelium, EphB3 mediated mechanism. A third possibility is that both signalling pathways may be involved and each was partially responsible for the likely enlargement of the epithelial buds.

An anterior prostate (AP) organ culture system was additionally used to investigate the role of EphB-EphrinB signalling in prostate development. The EphB3-Fc treatment, in the presence of testosterone, significantly decreased AP organ size by 47% after 6 days of culture (Figure 5.5B). This observation suggests a role for EphrinB signalling in AP development mediated by EphB signalling, perhaps via the EphB3 receptor. The decrease in AP organ size was consistent with the VP organ size reduction in response to EphB3-Fc treatment (Figure 5.1B). Collectively, these observations suggest that EphB3 has a role in prostate organogenesis.

The observation that neither of the EphrinB-Fc ligand treatments produced an effect in the AP organ culture was surprising as these treatments did in the VP organ culture (Figure 5.3) If an EphB receptor has a role in AP development, then one of its ligands may have a role in AP development. It is possible that another Ephrin ligand, maybe EphrinB3, is responsible. EphA1 and EphrinB1 are restricted to the epithelium and mesenchyme respectively during mammary branching morphogenesis (Kouros-Mehr and Werb., 2006). It is possible the EphA-EphrinB paracrine crosstalk occurs during branching morphogenesis in the development of organs such as the mammary gland and prostate. Furthermore, EphB receptors can bind to the EphrinA ligands. For example, EphA4 and EphrinA5 can interact with EphrinB2 and EphB2, respectively (Bouzioukh et al., 2007; Himanen et al., 2004). The EphB-Fc and EphrinB-Fc proteins may have inhibited any EphA signalling that was occurring in the VP and AP organs. However, EphA and EphrinA expression in the developing rat prostate was not investigated and any role for EphA signalling in rat prostate development was not examined.

Histological and immunohistochemical (IHC) analysis were used to ascertain if the addition of EphB-Fc and EphrinB-Fc proteins to VP organ cultures had an effect on the VP cellular morphology. The structure of the developing VPs was investigated using H+E staining. The epithelium and stroma were individually examined using the tissue specific markers, p63 (epithelium) and SMA (stroma). There was no effect on VP cellular morphology seen after H+E treatment (Figures 5.7 and 5.8) Furthermore, the EphB-Fc treatments appeared to have no effect on VP cellular morphology as revealed by p63 and SMA immunohistochemical staining (Figures 5.9 and 5.11). However, as a response to EphrinB-Fc ligand treatment, there appeared to be an increase in the size of individual buds as observed in the p63 and SMA immunohistochemical analysis (Figures 5.10 and 5.12).

6.4 The mechanisms of EphB-EphrinB signalling in the developing prostate

EphB receptor and EphrinB ligands were expressed in stromal and epithelial compartments during prostate organogenesis. Furthermore, the functional analysis of EphB signalling during rat prostate development suggests it has a regulatory role. During the WISH analysis, a potential EphB signalling mediator showed a mesenchyme expression pattern, RasD. The intracellular signalling mechanisms by which the EphB-EphrinB interactions transmit these effects have not been determined by this study. The identification of an effector of EphB signalling supports the idea that EphB signalling is active during prostate development. RasD is part of a protein family that may act as intracellular mediators of EphB-EphrinB signalling. In human umbilical vein endothelial cells, the Ras/MAPK cascade can mediate the intracellular signal downstream from EphrinB2-EphB interactions (Kim et al., 2002). The EphB2 positive DLD1 colon cell line, when stimulated with EphrinB1, activates Rho and Ras downstream (Riedl et al., 2005). These two studies suggest an association between EphB signalling and the Ras intracellular signalling. However, this association may be organspecific. However, restricted stromal expression of RasD in the prostate supports the notion that Ras GTPases mediate EphB signals in the stroma, during rat prostate development. Additionally, EphB2 can phosphorylate the R-Ras effector domain that directly inhibits cell adhesion (Dail et al., 2006). Thus, some effects of EphB signalling on prostate growth may be via cell adhesion.

An intracellular signalling mechanism that may be involved in EphrinB reverse signalling in the developing prostate are γ-secretase complexes, which produce a separate intracellular domain (ICD). ICDs have been reported to function as transcriptional activators or repressors in the nucleus (reviewed in Kopan and llagan., 2004). An EphrinB2 ICD is generated by a matrix metalloprotease (MMPs) and γ-secretase after binding to EphB2. The EphrinB2 activates Src by removing a repressor named Csk (Georgakopoulos et al., 2006).

Furthermore, the EphrinB1 ligand also has an ICD and is reported to have a role in F-actin regulation (Tomita et al., 2006). EphB receptors also undergo proteolytic cleavage. The extracellular domain of the EphB2 receptor is cleaved by calcium mediated ADAM10 protease cleavage. The EphB2 extracellular domain cleavage is enhanced by EphrinB2 binding (Litterst et al., 2007). The proteolytic cleavage of EphrinB1 and EphrinB2 ICDs may occur in prostate development. Although there is no direct evidence for it, EphrinB1/B2 ICDs may translocate to the nucleus of stromal (EphrinB1) and epithelial (EphrinB2) prostate cells. This could provide an additional intracellular mechanism for EphB-EphrinB signalling in prostate development. Antibodies raised against the EphrinB1 and EphrinB2 could be used in immunohistochemical approaches to investigate the possibility of EphrinB ICDs in prostate development.

Proteins that are phosphorylated by EphB receptors include adaptor proteins (Holland et al., 1997), R-Ras (Zou et al., 1999), the transmembrane proteoglycan syndecan-2 (Ethell et al., 2001), and the Rho-GEF Kalirin (Penzes et al., 2003). More recently, EphB4 signalling gas been reported to influence RhoA-mediated actin cytoskeleton reorganisation, which aids invasive melanoma cell migration in the mouse (Yang et al., 2006). EphB receptors have been shown to regulate the action cytoskeleton in spine formation (Henkemeyer et al., 2003). All the aforementioned molecules participate in the regulation of the actin cytoskeleton. The main cellular responses to EphB-EphrinB signalling are changes in cell morphology and positioning. Differences in cell shape are mediated by changes in the actin cytoskeleton. Taken together, this data suggests that EphB-EphrinB signalling during rat prostate development may elicit its effects via changes in the actin cytoskeleton.

Activation of the Eph receptors activation relies on ligand binding and dimerization. Therefore, Eph receptor tyrosine phosphorylation requires Ephrin ligands in either clustered or membrane-attached forms (Davis et al., 1994). Conversely, there is evidence to suggest that Eph receptor clustering, for example EphA3, does not require Ephrin ligand contact (Wimmer-Kleikamp et al., 2004). Given that two EphB receptors and two EphrinB ligands are expressed in prostate development, it is possible that the clustering of the EphB

receptors, dependent or not on EphrinB contact plays a role in their functionality. No experimental approach was taken as part of this thesis to determine if EphB/EphrinB clustering is required for correct prostate development.

Different splice variants of the EphB receptors and EphrinB ligands may play different roles with in the development of the prostate. Many members of the EphrinA ligand family have been reported to have splice variants, such as EphrinA1, A3, and A5 (Lai et al., 1999; Finne et al., 2004). EphB2 has been reported to have three different transcripts of in three sizes of approximately 4, 5, and 11 kb. The variety EphB2 splice variants may represent its functional heterogeneity in different tissues (Tang et al., 1998). It is unclear which of the EphB2 splice variants plays a role, if any, in prostate development as no experimental approach was undertaken with regards to splice variants. Additionally, the SAGE data in Figure 4.1 revealed no information with regards to the abundance of an EphB2 transcript in prostatic tissues. It is possible the high levels of EphB2 mRNA seen in Figures 4.1 and 4.3 contained more than one EphB2 mRNA, based on the evidence from the Tang paper.

The EphB and EphrinB genes maybe targets for other signalling pathways. The Notch pathway is important in prostate organogenesis with regard to both the epithelium (Wang et al., 2006) and the mesenchyme (Orr et al., 2009). EphrinB2 is a direct Notch target during ventricular myocardium development (Grego-Bessa et al., 2006). Notch signalling may regulate the expression of EphrinB2 or other EphB-EphrinB genes in the developing prostate. Members of the FGF family, such as FGF7 (Sugimura et al., 1996) and FGF10 (Thomson and Cunha., 1999) are important for prostate organogenesis. Furthermore, the FGF receptor activates EphrinB1 in *Xenopus* embryos (Chong et al., 2000) and FGF signalling modulates EphrinB1 signalling in retinal progenitor cells (Moore et al., 2004). As FGF and EphrinB1 signalling both have functions in rat prostate development, they may interact.

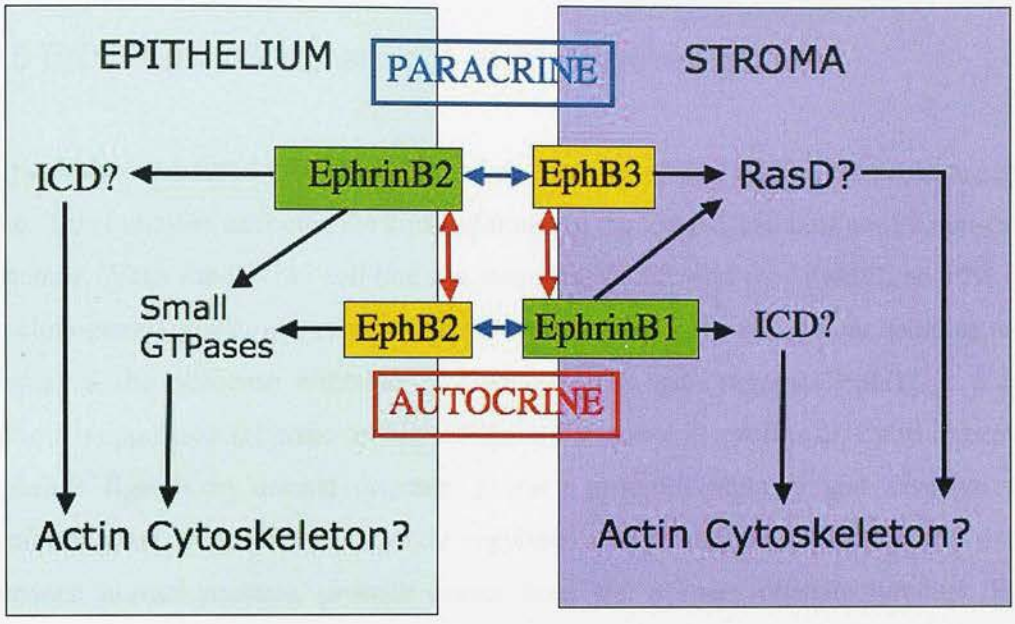

Figure 6.1 EphB receptor and EphrinB ligand expression in the rat ventral prostate during development and possible mechanisms of action. Both paracrine and autocrine EphB-EphrinB interactions are possible due to the expression patterns of the EphB2/B3 receptors and the EphrinB1/B2 ligands. RasD is expressed in the stroma. It is possible that this intracellular mediator is part of the EphB forward signalling pathway. Any small GTPases could mediate EphB-EphrinB signalling in the epithelium. Alternatively, the intracellular domains (ICD) of the EphrinB1/B2 ligands may be responsible for the transmission of the EphrinB reverse signal. The most likely mechanism effected used to elicit the influence of EphB-EphrinB signalling on prostatic cell proliferation and positioning is the regulation of the actin cytoskeleton.

All of the EphB-EphrinB expression data, possible interactions and intracellular mediators are summarised in Figure 6.1. The role of EphB-EphrinB signalling in the prostate has been discussed under the context of development. There may be role for EphB-EphrinB signalling in prostate cancer.

6.5 EphB-EphrinB signalling in prostate carcinogenesis

A nonsense mutation in the EphB2 gene has been reported in the DU145 prostate cancer cell line. This mutation truncated the kinase domain of the EphB2, and produced a non-functional receptor. When the DU145 cell line was transfected with wild type EphB2, an 80% decrease in clonogenic growth was reported. Furthermore, 8% of prostate cancer samples examined displayed the nonsense mutation. Collectively, this data suggests EphB2 is a potential tumour suppressor (Huusko et al., 2004). An expression profile of EphB receptors and EphrinB ligands in normal prostate, primary prostatic tumours and invasive prostatic tumours was conducted. This study reported a large degree of differential expression between normal prostate, prostate cancer lines and primary prostate tumours. EphB2 is expressed at similar levels in normal prostate and primary prostatic tumours but was downregulated in four prostate cancer cell lines. EphB3 was expressed in normal prostate but not in primary tumours. However, EphB3 expression was upregulated in four prostate cancer cell lines suggesting EphB3 may have a role in more advanced prostate cancer. This could suggest epithelial-mesenchymal transition (EMT) since EphB3 is expressed in the mesenchyme. EphrinB1 was expressed in normal prostate, primary tumours and two prostate cancer cell lines at similar levels. EphrinB2 expression was similar in normal prostate and primary tumours but was downregulated in three prostate cancer cell lines (Fox et al., 2006). These observations suggest EphB signalling has a function in the progression of prostate cancer. The study discussed here did not attempt to investigate any role of EphB-EphrinB signalling in prostate carcinogenesis.

6.6 Summary

A WISH analysis found that 13 mRNAS were expressed in the mesenchyme of the rat prostate during development. One of these transcripts encodes the EphB3 receptor. Furthermore, the EphrinB1 ligand was determined to be expressed in the mesenchyme during rat prostate development. The EphB2 receptor and the EphrinB2 ligand were predominantly expressed in the epithelium in rat prostate development. Both receptors and ligands were expressed at their most abundant levels in the VP during prenatal and early postnatal development. Using *in vitro* VP organ cultures and exogenous recombinant protein treatments, it was determined that increased EphrinB reverse signalling, decreased EphB forward signalling or a combination of both decreased VP organ size. Conversely, it was determined that decreased EphrinB reverse signalling, increased EphB forward signalling or a combination of both increased VP organ size. These observations show that EphB-EphrinB signalling was involved in the regulation of rat prostate development. The results observed from the addition of EphrinB-Fc to the VP cultures suggest EphB-EphrinB signalling could regulate the size of developing VP epithelial buds. This regulatory signalling could come from either epithelium in a juxtacrine/autocrine manner or by paracrine signalling from the stroma. These pathways are likely to affect the actin cytoskeleton, and consequently change the cell morphology and positioning of the prostate epithelium and stroma.

This thesis determined the members of the EphB receptor and EphrinB ligand families were expressed in both the mesenchyme and epithelium of the rat prostate during development. Furthermore, functional evidence was ascertained to suggest a regulatory role for EphB-EphrinB signalling in rat prostate organogenesis.

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