THE ROLE OF GLIAL CELL LINE- DERIVED
NEUROTROPHIC FACTOR SIGNALLING PATHWAY
IN MAMMALIAN ORGANOGENESIS

By

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1997
To Jonny
Abstract

Ret is a receptor tyrosine kinase which binds GDNF, in partnership with GDNFRα, a glycosylphosphatidylinositol-linked cell surface receptor. Ret and GDNF have implicated in branching morphogenesis of the metanephros. Ret is expressed by the ureteric bud and its derivatives, whilst GDNF is expressed by the mesenchyme. Null mutant mice for either gene exhibit severe dysgenesis or agenesis of the kidney and ureter. However, their role in branching morphogenesis of other organs is unclear.

This study has further examined the expression pattern of ret, GDNFRα, and GDNF during mouse development, with particular reference to organs that develop through branching morphogenesis. The pathway was expressed in both the metanephros and lung, but in differing spatial and temporal patterns in the mesenchyme and epithelia, but only in presumed ganglia cells of the submandibular gland. Ureteric bud and nephron formation were accelerated in organ cultures containing exogenous GDNF, with the ectopic growth of supranumary ureteric buds; the opposite effect was observed in cultures containing neutralising antibodies. Development of the lungs and submandibular glands were unaffected under these conditions. Hence, GDNF is a mesenchymal derived factor which causes outgrowth and development of the ureteric bud epithelia.

The role of ret activation in monoclonal cell lines was also investigated. Renal cells lines were stably transfected with ret and a ret oncogene, ret/PTC2. They were assayed for changes in proliferation, and growth morphology in three dimensional cultures. The activation of ret, and the presence of ret/PTC2, caused a significant reduction in cellular proliferation as well as causing MDCK cells to produce 'spikes' when grown in collagen gels. Together these results suggest a morphogenetic role for ret in development.

Finally, a three dimensional culture system has been described. This may be used in future work to assay the effects of other factors on nephrogenesis.

* Glial cell line-derived neurotrophic factor

** Glial cell line-derived neurotrophic factor receptor α
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<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethlypyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dig</td>
<td>digoxigenin</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell derived neurotrophic factor</td>
</tr>
<tr>
<td>GDNFR(\alpha)</td>
<td>glial cell derived neurotrophic factor receptor alpha</td>
</tr>
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<td>H and E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HGF/SF</td>
<td>hepatocyte growth factor/scatter factor</td>
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<td>kilodaltons</td>
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<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Lennox broth</td>
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LIF  leukaemia inhibitory factor
LMP  low melting point
M    Molar ie. concentration in moles per litre
ml   millilitre
mg   milligram
mRNA messenger RNA
NGF  nerve growth factor
NTP  nucleotriphosphate
OD   optical density
PAX  paired box
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDGF platelet-derived growth factor
PFA  paraformaldehyde
poly-A poly-adenylated ribonucleic acid
RNA  ribonucleic acid
RNase ribonuclease
r.p.m. revolutions per minute
RT   reverse transcription
RT-PCR reverse transcription polymerase chain reaction
SDS  sodium dodecyl sulphate
SSC  sodium chloride/sodium citrate buffer
TAE  tris acetate EDTA electrophoresis buffer
TE   tris EDTA
TEMED N,N,N',N'-tetramethylethylenediamine
TGF α and β transforming growth factor alpha and beta
UTP  uridine triphosphate
TNFα tumour necrosis factor alpha
UV   ultra violet
x    times
X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactosidase

All units are SI (Système International)
1. Introduction

1.1 Mesenchymal and epithelial interactions during development

Epithelia and mesenchyme are two distinct cell types found in virtually every organ of the metazoan body. Epithelia are composed of closely associated, largely immobile cells, whereas mesenchyme contains more mobile, fibroblastic-like cells that form loosely associated cell agglomerations. Mesenchymal-epithelial interactions are intrinsic to many of the processes involved during development. During normal development, transitions between epithelia and mesenchyme occur, for example in gastrulation, and mesenchyme can differentiate into new epithelia, as can be seen in nephron development. Such transitions are not confined to development. In particular, the loss of epithelial character in malignant carcinomas, resulting in the appearance of invasive, motile cells, is of major importance in tumour progression (Byers et al., 1994). The second type of interaction occurring between epithelial and mesenchymal cells is the process of induction, the mechanism by which signals generated by one group of cells controls the fate of neighbouring cells (Spemann and Mangold, 1924; Gordon, 1989). Inductive interactions involve two primary components: a signal that is generated by the inducing cell, and a receptive system that, directly or indirectly, controls gene expression in the responding cell. The ligands that constitute inductive signals can be anchored to the cell surface or secreted from cells. Thus, the extent of induction can be controlled by regulating ligand production, or by limiting its range of action. The competence of cells to respond to the ligand also contributes to the extent of induction. Competence may be controlled by modifying the expression or function of the appropriate receptors, the intracellular signal transduction pathway, or the transcription of target genes.

Branched tubular epithelial structures are found in most animals and function to transport gases and liquids in the body. These organs arise through mutual induction of epithelial and mesenchymal cell lineages. After many years of intensive study, many of the inductive signals and receptors involved in branching morphogenesis remain to be identified, although, more recently, light has been shed onto the molecular control of these processes. Work within this project clarifies the involvement of the ret-GDNF (glial cell line-derived neurotrophic factor) signalling pathway in the branching morphogenesis of the mouse metanephros, the direct precursor of the adult kidney.

Work in the early 1950s by Clifford Grobstein demonstrated that, once separated from each other, both the epithelia and the mesenchyme of the metanephros, lung, and
submandibular salivary gland (hereafter referred to as the salivary gland) cannot undergo any degree of differentiation, and die within a few days (Grobstein, 1953). More recently, several groups have been able to culture the epithelia of the metanephros, lung, and salivary gland in combinations of basement membrane matrix (Matrigel™), or collagen type I gels, when supplemented with specific growth factors (Perantoni et al., 1991; Nogawa and Ito, 1995; Nogawa and Takahashi, 1991), thus, these growth factors are implicated in the molecular control of the fate of these epithelia.

Initial recombination studies by Grobstein illustrated that, while some epithelia are highly promiscuous as to their dependence on particular mesenchyme, for example the lung (Deucher, 1975), others, such as the metanephric epithelial bud, are not (Grobstein, 1955). This work led to the hypothesis that there are mesenchymal specific (or instructive) factors, and mesenchymal common (or permissive) factors (Grobstein, 1967). The distinction between an instructive or permissive inductions was emphasised by Saxen. A truly instructive induction is one in which completely naive cells are instructed which of several possible responses to follow, according to the nature of the inducing signal. Conversely, a strictly permissive induction is one in which a cell has only two options: to continue along its current path of differentiation, or to be diverted into the only other pathway that it is capable of following. Most inductions fall somewhere between the two extremes, but it is important to appreciate that nearly all approximate more closely to the permissive, rather than the instructive type (Saxen, 1989). More recently, work by Kispert et al. demonstrated that ureteric bud epithelium can be induced to undergo branching morphogenesis when recombined with lung mesenchyme (Kispert et al., 1996). However, the pattern of branching in these cultures is more organotypic to the lung bronchial tree rather than the kidney collecting duct system. Studies such as these, along with results from this project, has led to a refining of Grobstein's original hypothesis to one which includes the idea that it is the spatial and temporal expression of genes, as well as the concentration of the gene products, that leads to their instructive or permissive functions within specific organs.

1.2 Techniques for studying branching morphogenesis in vitro and in vivo

There are many techniques routinely used to define the mechanisms of branching morphogenesis. They fall under the two main headings of descriptive and functional studies. Descriptive studies examine basic anatomy and correlate this with gene and protein expression patterns within the developing organ. From these comparisons, hypotheses can then be drawn and tested using functional studies.
1.2.1 Descriptive studies

1.2.1.1 Microscopy

Both electron and light microscopy are routinely used in anatomical studies. Microscopy has been used in this project to examine the finite cell morphology of an in vitro model for metanephric mesenchymal development, allowing the visualisation of tight junctions, mitotic bodies, and the absence of necrosis or apoptosis (Towers et al., 1994). Qiao et al. demonstrated, using viral cell labelling and electron microscopy, that the metanephric blastema contributes to the collecting duct system as well as differentiating into the nephrons (Qiao et al., 1995).

1.2.1.2 Expression studies

There are many techniques open to the investigator that enable the study of gene and protein expression patterns. Due to the small amounts of RNA available, the highly sensitive methods of RT-PCR and RNase protection assays are most appropriate for the study of gene expression in embryonic organs. In situ hybridisation, using digoxygenin (dig) or \(^{35}\)S-labelled riboprobes, can be used on whole mount and sectioned specimens. In situ hybridisation, using dig-labelled riboprobes, is arguably the more sensitive of the two techniques due to the layering effect of the riboprobes, antibodies, and colour reaction (Wilkinson, 1997). Whole mount preparations can be visualised intact, and then mounted in albumin-gelatine in order to be sectioned. This allows the localisation of any signal to a particular cellular population.

Immunohistochemistry allows the localisation of protein by the binding of specific antibodies tagged with a fluorescent, or colour reaction marker. Not only does this demonstrate the translation of the RNA, but it can also define the specific localisation of the protein within the cell or extracellular milieu.

1.2.2 Functional studies

Functional studies can be aimed at various levels of investigation, ranging from primary cell culture to transgenic techniques.

1.2.2.1 Transgenic animals

Transgenic technology utilises homologous recombination to replace the existing genomic gene, with a genetically engineered gene. This may be in the form of a null mutant (Kreidberg et al., 1993), or fused to a ubiquitous promoter, thus resulting in a dominant gain of function (Dressler et al., 1993), or a dominant negative effect (Dumont et al., 1994) Early
embryos are reconstituted with genetically engineered embryonic stem (ES) cells (Liu et al., 1993). Homozygous mice can then be generated by the breeding of the founder generation. Many transgenic animals exhibit a normal phenotype in organs where the wild type gene is known to be expressed in vivo (Mendelsohn et al., 1994). Important functions are often controlled by more than one gene, meaning that in the event of a mutation, the animal will still develop normally. Thus, many pathways work as the 'belt and braces' of development, being functionally redundant in normal development. Cross breeding between transgenic mice can lead to animals harbouring multiple mutations to help overcome the potential problem of functional redundancy within biological pathways (Stein et al., 1994). Some phenotypes have been shown to be strain dependent, suggesting the presence of modifier genes, which further complicates the analysis of the phenotype (Threadgill et al., 1995).

The roots of the current project lie in the phenotype of the homozygous null mutant mouse for the receptor tyrosine kinase, ret (Schuchardt et al., 1994). These animals display a range of renal abnormalities from blind ending ureter, to dysplastic, non-functioning metanephric rudiments. In 1996, this phenotype was replicated in mice harbouring a null mutation for the ligand to ret, glial cell line-derived neurotrophic factor (GDNF) (Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996).

The phenotype of transgenic animals suggests roles of the manipulated gene(s) in the development of the affected organ(s). However, these studies are not definitive. Firstly, as previously discussed, many animals have been surprisingly free of malformations due to the functional redundancy within many pathways. Secondly, complete agenesis of an organ can only suggest a functional role of the gene in the initiation of development, but provides no insight into the potential role of the gene in further development or function of the adult organ. Furthermore, malformations may be secondary to the effects of a primary lesion. For example, it has been suggested that pulmonary hypoplasia can be caused by many different factors, such as lack of space for the lungs to grow, inhibition of the necessary respiratory movement during intrauterine life, or a lack of amniotic fluid (specifically associated with renal agenesis in Potter's syndrome) (Page and Stocker, 1982).

1.2.2.2 Whole organ cultures

Organ cultures of the metanephros, or other embryonic rudiments, can overcome many of the difficulties described above. They have been used ever since their initial description in the 1950s by Grobstein, who demonstrated that organ rudiments can be grown and manipulated in vitro (Grobstein, 1953). The cultures can be manipulated in the following ways:

- Addition of antisense oligonucleotides, blocking the translation of the RNA (Rothenpieler and Dressler, 1993)
• Addition of specific antibodies which bind to the functional domain of the protein, thus blocking its activity (Rogers et al., 1992),
• Addition of exogenous growth factors. (Bard and Ross, 1991)

Organs may be removed at various stages of development and, once in culture, can be grown in a defined (without serum) or a semi defined (with serum) medium. The development of the organs can then be observed and analysed over several days.

1.2.2.3 Culture of isolated epithelium or mesenchyme

The interactions between the epithelia and mesenchyme are highly complex and bidirectional, with the development of one being dependent on the development of the other. For this reason, it is not always obvious which cell lineage the factor is primarily affecting. For example, in the ret null mutant mice, neither the epithelial nor the mesenchymal components develop normally, in vivo (Schuchardt et al., 1994), although it has been demonstrated in organ culture that the null mutation primarily affects the development of the ureteric bud epithelium which, in turn, fails to induce the mesenchyme (Schuchardt et al., 1996). To remove this ambiguity, the mesenchyme and epithelia can be dissected apart and cultured alone. The cells can then be grown in two or three dimensional cultures, where the specific effects of individual factors can be assessed.

1.2.2.3.1 Culture of isolated epithelium

Epithelium from the metanephros, lung, or salivary gland, can be grown in three dimensional cultures of collagen type I gels (supplemented with fibroblast growth factor, FGF) or of Matrigel™ (supplemented with epidermal growth factor, EGF) (Perantoni et al., 1991; Nogawa and Ito, 1995; Nogawa and Takahashi, 1991). Under these conditions, the epithelia grow and branch in a similar fashion to that seen in vivo, except that the metanephric epithelium hardly develops at all. These culture systems can be manipulated, in vitro, to further elucidate the factors involved in branching morphogenesis. The ureteric bud epithelium from the mouse has proved more difficult to grow in three dimensional culture, although it has recently been shown to survive for a few days as a primary monolayer under certain conditions (Towers et al., 1997). These protocols allow further dissection of the molecules involved in the ureteric bud development.

1.2.2.3.2 Culture of isolated uninduced mesenchyme

There have also been many studies involving the culture of isolated embryonic mesenchyme, most of these having centred on the culture of uninduced metanephric mesenchyme. The development of this tissue will be described in full later, but for now it is
important to note that upon induction by the ureteric bud, the metanephric mesenchyme not only proliferates, but also undergoes a mesenchymal to epithelial transition. Original heterologous recombination studies across a glass clot interface demonstrated that metanephric mesenchyme can be induced to develop into nephrons when cultured with embryonic salivary gland epithelium or with dorsal embryonic spinal cord (Grobstein, 1955; Grobstein, 1956). Under these conditions, no other mesenchymal tissue has been shown to develop nephrons, suggesting that this induction is a permissive effect. Induction is blocked if the cultures are set up across filters with pore sizes < 0.1 \( \mu \text{m} \) (Saxen and Lehtonen, 1978). This led to an initial hypothesis that pores of this size block the formation of neuronal processes which are the mechanism of induction. However, the accepted theory is now based upon observations made by Karavanova et al., who suggested that such small pore sizes do not provide sufficient support for the mesenchyme. They demonstrated that filters with pore sizes > 0.1 \( \mu \text{m} \), coated with collagen, proved good attachment and enhance mesenchymal differentiation in response to conditioned medium from a ureteric bud cell line (Karavanova et al., 1996).

1.2.2.4 Cell lines

The metanephric mesenchyme does not consist of a homologous population of cells. As well as nephron precursors, it contains stem cells (Bard et al., 1994), and endothelial precursors (Loughna et al., 1997). For this reason, many studies are carried out on clonal cell lines to define the exact mode of action of a particular factor on a particular cell type. In Chapter 5 of this thesis, clonal renal cell lines have been used to investigate the role of the ret-GDNF signalling system in metanephrogenesis.

It has been demonstrated that some adult epithelial cells remain responsive to inductive influences from connective tissue (Modges, 1982), differentiated, cultured epithelial cells can be used to investigate the possible morphogenetic role of diffusible stromal signals. The Madin-Darby canine kidney (MDCK) cells retain several anatomical and functional properties to those of kidney collecting ducts (as discussed in Montesano et al. 1991), and have been shown to generate either spherical cysts (McAteer et al., 1987), or tubule-like structures (Montesano et al., 1991), in vitro. For these reasons, they have been the subject of many studies connected with renal epithelial morphogenesis, including the current one.

1.3 Development of the metanephros

Part of this thesis involves a comparative study between three organs that develop by branching morphogenesis; the metanephros, the lung, and the salivary gland. For this reason, the development of all three organs are discussed in their own right, but it should be noted that
the focus of this work was the development of the mammalian metanephros. The developing metanephros is an attractive model system for examining organogenesis, as it represents a 'snap-shot' of its development. Early elements can be seen at the peripheral nephrogenic zone whilst immature aggregates are observed adjacent to them and maturing nephrons are located nearer the medulla, or centre of the organ.

Adult kidneys are responsible for several functions, including regulation of the amount of water passed from the body, filtration of nitrogen-containing waste from the bloodstream, and maintenance of the correct balance of electrolytes. They are therefore essential for life, and in order to function normally, must have first undergone a complex program of development, known as nephrogenesis.

The development of the embryonic excretory system involves the transient formation, and subsequent regression, of vestigial primitive systems in a caudocranial sequence. The intermediate mesoderm on either side of the dorsal body wall gives rise to three successive nephritic structures of increasingly advanced design. The first of these structures, the pronephros, develops on embryonic day 9 (E9) of the mouse and consists of a small group of segmental nephrotomes. As these cranial nephrotomes regress, they are succeeded by a pair of elongated mesonephroi, which develop in the thoracic and lumbar regions. The earliest marker for the mesonephric mesenchyme is the expression of lim-1, a member of the hox gene family, at E8.5 (Fugii et al., 1994). The mesonephroi are functional, having complete, although simple, nephrons. They are drained by a pair of mesonephric (Wolffian) ducts, which grow caudally to open into the posterior wall of the primitive urogenital sinus. The Wolffian duct passes a dense mesenchymal blastema at E10. This metanephric mesenchyme expresses the transcription factor wt-1, which is currently the earliest known molecular marker of the metanephric blastema (Mundlos et al., 1993). By E11, a ureteric bud has sprouted from each of the Wolffian ducts, and has penetrated its metanephric blastema, marking the first morphogenetic event in metanephric development. The ureteric bud and the metanephric blastema then undergo mutual induction, giving rise to the adult metanephros (Figure 1.1). The work of Grobstein, discussed earlier, provides the generally accepted view that the ureter, renal pelvis, calyces and collecting tubules arise from the ureteric bud epithelium, whilst the nephrons and stromal tissue arise from the metanephric mesenchyme. However, a recent, and controversial, study by Qiao et al. suggests that retrovirally tagged ureteric bud or mesenchymal cells can have a minor contribution to the nephrons or collecting duct system, respectively (Qiao et al., 1995). It is therefore possible that the two cell lineages exhibit a higher degree of plasticity than was previously thought.
1.3.1 Development of the ureteric bud

When the ureteric bud first contacts the metanephric blastema, its tip expands to form an initial ampulla. Under the inductive influences of the metanephric mesenchyme, the ureteric bud grows and bifurcates. As development of the metanephros continues, the number of ampullae increase and the branches start to coalesce to form the major and minor calyces extending from the renal pelvis. Each minor calyx drains a tree of collecting ducts into a renal pyramid which then converges to form the renal papilla of the adult organ. This pattern of development establishes distinct medullary and cortical collecting duct segments that are lined by two major cell types; principal (potassium ion handling cells), and intercalated (hydrogen ion handling cells). Work within this project provides evidence to suggest that GDNF is one of the mesenchymally derived factors, which not only induces initial evagination of the ureteric bud, but also stimulates its further development.

1.3.2 Development of the nephrons

After its initial invasion, the ureteric bud begins to induce the mesenchyme. The primary effect of this induction is the prevention of apoptosis (Koseki et al., 1992). This is followed by a proliferative burst and condensation of the mesenchyme surrounding each branch tip. These are the primary events in a cascade of sequential change, including alterations in gene expression and cellular morphology (Davies and Garrod, 1995). Each condensed aggregate epithelialises and elongates to form a comma shape, which further elongates and turns back on itself to become the S-shape body. The tail of the S becomes the glomerular region, whilst the other end of the S becomes the distal portion of the nephron, the tip fusing to the growing ureteric bud-derived collecting duct. The glomeruli are invaded by capillaries, with the inner epithelial visceral layer finally differentiating into the podocytes which surround these capillaries.
Figure 1.1 Reciprocal induction in the development of the mammalian metanephros

As the ureteric bud enters the metanephric mesenchyme, the mesenchyme induces the bud to branch. At the tips of the branches, the epithelium induces the mesenchyme to aggregate and cavitate to form the renal tubules.

Figure taken from Gilbert (1988).
samples failed to progress into the later stages of the nephrogenic development. The temporal expression patterns of many genes, such as wnt-4, and bone morphogenetic protein 7 (BMP7), suggest that they are involved in this multiphasic pattern of development, as discussed in more detail later.

1.3.4 Development of the blood supply, neuronal, and stromal cells

Alongside the differentiation of the mesenchyme into the nephrons, is the continuing proliferation of the stem cell population. These cells are a subset of the uninduced mesenchymal cells, but are distinct from them. They are located at the periphery of the organ, and can be identified by a number of markers. They express the trk-B nerve growth factor (NGF) receptor, as opposed to the low affinity NGF receptor expressed by the mesenchyme. They also express hox-c.9 and pax, markers which are not expressed by the uninduced mesenchyme, as discussed in Bard et al. (1994). Stem cells may follow one of two possible differentiation pathways. Some will condense into small aggregates and be incorporated into the developing nephron, whilst the remainder will differentiate to give the stromal cells. Stromal cells have been shown to be of vital importance in the development of the metanephros. Mice harbouring a null mutation in the BF2, a transcription factor gene expressed in the stromal cell lineage, develop small, fused, and rotated kidneys (Hatini et al., 1996).

Paralleling these events is the differentiation of the blood supply and other functional components, such as the rennin-producing cells in the juxtaglomerular apparatus with their associated neural connections. At the time of ureteric bud outgrowth, the metanephric mesenchyme contains no patent capillaries. It was previously accepted that the kidney is vascularised by angiogenesis, a process by which the metanephros is invaded by capillaries from the outside (Sariola et al., 1983). However, work from our laboratory has shown this not to be the case. Loughna et al. (1997) demonstrated that endothelial precursors are present in the early metanephric mesenchyme. When rudiments were grown in an experimental environment which mimics the in vivo situation, these precursor cells were able to differentiate and give rise to the glomerular capillaries found in the adult kidney. The neurones of the kidney, which regulate blood flow and secretory function, arise from neural crest cells that invade the metanephroi early in their development (Sariola et al., 1988). These neurones can be found at the tips of the metanephric tissue caps during the phase of nephron induction, with in vitro experiments suggesting that they may play a role in this induction.(Saxen and Lehtonen, 1978)
1.3.5 Apoptosis in the developing metanephros

As well as proliferation and differentiation, there is a degree of apoptosis which is known to occur during normal development. This process is distinct from necrosis (Kerr et al., 1972), and serves to eliminate unwanted cells. During necrosis, the cell usually swells and lyses, spilling its cytosolic components into the extracellular space, which elicits an inflammatory response. In apoptosis, the cell and its nucleus shrink, and often fragment. These fragments (called apoptotic bodies) are rapidly phagocytosed by either neighbouring cells or macrophages before there is any leakage. In the developing metanephros, up to 3% of cells in some areas of the mesenchyme and its derivatives are apoptotic (Koseki et al., 1992), and these levels can be reduced by EGF (Coles et al., 1993). Most of this apoptosis is found in the nephrogenic zone. In this area, 60% of apoptotic cells are found among the metanephric mesenchymal cells that were close to, but not in, nephrogenic structures. The remainder have been found to be in the epithelial cells of the early nephron tubules (Coles et al., 1993). By adulthood, the levels of apoptosis are negligible. It is therefore assumed that apoptosis is a normal component of nephrogenesis. The proto-oncogene \textit{bcl-2} inhibits cell death, and null mutations in this gene lead to the development of hypoplastic kidney rudiments with increased levels of apoptosis (Sorenson et al., 1995).

1.3.6 Molecular control of nephrogenesis

1.3.6.1 Secreted factors and their receptors

GDNF is expressed by the developing renal mesenchyme (Hellmich et al., 1996) and its receptor, ret, is expressed by the branching tips of the ureteric bud (Pachnis et al., 1993). These two proteins have been shown to be of great importance in the development of the metanephros and will be discussed in more detail later. Hepatocyte growth factor (HGF) is a cytokine secreted by the renal mesenchyme. Its receptor, met, is expressed by both the ureteric bud and nephron epithelia as well as the renal mesenchyme (Sonnenberg et al., 1993; Woolf et al., 1995), suggesting a role for HGF in the development of both cell lineages. Several studies have implicated these molecules, both in the growth and branching of the ureteric bud in whole metanephric organ culture (Woolf et al., 1995; Davies et al., 1995), and in the culture of MDCK cells in three dimensional gels (Montesano et al., 1991). These studies have shown that HGF can act as a mitogen or morphogen, depending on the culture conditions used, with the effect, in MDCK cells, being blocked by transforming growth factor \(\beta\)-1 (TGF\(\beta\)-1) (Santos and Nigam, 1993). Interestingly, HGF or met transgenic mice exhibit no renal phenotype (Schmidt et al., 1995; Vehara et al., 1995; Bladt et al., 1995), although the embryos
die at E13-14 from placental defects, suggesting that the HGF-met signalling system is possibly more important in later stages of renal development.

EGF and insulin-like growth factor (IGF) have also been shown to play a part in the orchestration of ureteric bud development. EGF enables a limited degree of branching of the isolated ureteric buds in three dimensional culture (Perantoni et al., 1991), and causes epithelial hyperplasia in organ culture (Pugh et al., 1995). Conversely, blocking antibodies to IGF I and II inhibits bud growth in organ cultures (Rogers et al., 1991). IGF II and its type I receptor are negatively regulated by the transcription factor wt-1 (Xie et al., 1994), as discussed later. Another receptor tyrosine kinase, ros, is expressed by the ureteric bud at E11, and thereafter throughout development in the branching tips of the bud. The ligand for ros has not yet been identified, but studies using antisense oligonucleotides have demonstrated a role for ros in the development of the ureteric bud, these cultures exhibiting impaired bud growth and morphology (Kanwar et al., 1995). However, in a similar fashion to that described for HGF, the null mutant mice for ros exhibited normal nephrogenesis (Sonnenberg-Riethmacher et al., 1996).

The inducing factor(s) present in the embryonic spinal cord, the most potent tissue inducer of nephron formation in vitro, has been shown to not be freely soluble (Herzlinger et al., 1994). This observation puts the wnt family members high on the list of possibilities. The wnt proteins are secreted, matrix binding glycoproteins that are tightly associated with the cell surface. They are thought to act in a paracrine manner, with their receptors being unknown (Nusse and Varmus, 1992). NIH 3T3 cells transfected with wnt-1 and recombined with isolated metanephric mesenchyme can induce nephron formation in a similar manner to that seen by the spinal cord (Herzlinger et al., 1994). In addition, wnt-1 is expressed by the spinal cord in an identical pattern to its inductive ability (Gavin et al., 1990). However, wnt-1 has not been shown to be expressed by the ureteric bud, although another family member, wnt-11 has been. Wnt-11 is expressed by the Wolffian duct at E10, and subsequently, as development proceeds, is restricted to the branching tips of the ureteric bud (Kispert et al., 1996). The homozygous Danforth short tailed mouse exhibits severe truncation of the urogenic axis, including hypoplastic or agenic kidneys. This is due to a lack of ureteric bud outgrowth, owing to an insufficient proximity of the Wolffian duct and metanephric blastema (Phelps and Dressler, 1993). Interestingly, these mice do not express markers of early renal induction, including wnt-11. Furthermore, wnt-11 transfected PC12 cells caused the upregulation of ret (Zheng et al., 1996). However, this is unlikely to be important in the early events of metanephros development, as ret is already expressed by the Wolffian duct at the time of reported wnt-11 expression (Pachnis et al., 1993).

Another member of the wnt family, wnt-4, is expressed in the induced mesenchyme and not in the ureteric bud (Stark et al., 1994). Null mutations for wnt-4 result in mice with
severely malformed dysplastic kidneys. The development of the nephrons is arrested after the initial induction; no tubules are present although there is a limited degree of proliferation (Stark et al., 1994). This finding is consistent with a defect in mesenchymal to epithelial conversion, and adds weight to the hypothesis that the development of the nephrons is a multiphasic process.

Other factors are known to become more important in the later stages of nephrogenesis. BMP7 is a member of the TGFβ family of cytokines. It is expressed by the ureteric bud during development, and is present in ureteric bud conditioned media (Karavanova et al., 1996). Null mutant mice display normal nephrogenesis up to E14. Subsequently, the co-ordinated program of cell growth and differentiation is not maintained, with decreasing levels of ureteric bud branching and corresponding numbers of condensates. However, BMP7 has been shown to have inductive capabilities, being able to induce metanephric mesenchyme in the absence of the ureteric bud (Vukicevic et al., 1996). This suggests that the role of BMP7 becomes progressively more important as the metanephros develops.

Some growth factors have been reported to have an inhibitory effect on nephrogenesis. The ES-cell inhibition factor, LIF, is a cytokine that has an inhibitory effect on the later stages of nephrogenesis, although it affects only the ability to form tubules, and not the initial aggregation stage (Bard and Ross, S.A. 1991), thus supporting the hypothesis of multistep nephron development. However, it has not been demonstrated to be present in the metanephros in vivo. The addition of TGFβ to metanephroi, in culture, leads to the inhibition of tubule formation, whereas the addition of blocking antibodies accelerates tubule formation (Rogers et al., 1993). Activin, another member of the TGFβ family, is also found to be expressed along with its receptors in the developing kidney. Activin treated metanephroi exhibit retarded branching of the ureteric bud, which is abnormally short (Ritvos et al., 1995). Tumour necrosis factor alpha (TNFα) is a classical inflammatory cytokine, which has been shown to be an endogenous negative regulator of nephrogenesis, although it is unknown which cells express the factor in vivo. The addition of exogenous TNFα blocks nephrogenesis in whole organ culture (Cale et al., 1996). Together, these inhibitory factors probably control the final size of the adult organ. TGFβ, activin, and TNFα are all known to be expressed in the developing metanephroi, although their expression has not been localised to specific cell lineages.

1.3.6.2 Transcription factors

Lim-1, a member of hox gene family, encodes a protein containing a DNA binding motif, and is expressed in the intermediate mesoderm of mice at E8.5 (Fugii et al., 1994). Null mutant mice for lim-1 lack urogenital, as well as head structures (Shawlot and Behringer, 1995).
Another important transcription factor is encoded by the Wilm's tumour gene (wt-1). This is expressed during metanephric development in the uninduced mesenchyme, and is known to be a transcriptional repressor (Xie et al., 1994; Weidner et al., 1990). After birth, low level expression is restricted to the glomerular podocytes (Mundlos et al., 1993). In mice expressing a null mutation in wt-1, there is no ureteric bud outgrowth from the Wolffian duct (Kreidberg et al., 1993). This suggests that wt-1 is involved in the transcriptional control of inductive factors emanating from the mesenchyme, which subsequently causes the outgrowth of the ureteric bud. In Chapter 4, it is demonstrated that GDNF is one of these mesenchymally derived inductive factors. Its expression in the metanephric mesenchyme coincides with that of wt-1, see Chapter 3, although, it is currently unknown whether wt-1 is directly involved in the control of GDNF expression.

The transcription factor pax-2 is up regulated as the mesenchyme is induced (Dressler and Douglas, 1992), but is down regulated as the mesenchymal condensates differentiate into nephrons. Pax-2 is also expressed in the tips of the branching ureteric bud (Winyard et al., 1996). Transgenic mice that over express pax-2 exhibit epithelial hyperproliferation and cyst formation (Dressler et al., 1993). Conversely, genetic ablation of a single pax-2 allele causes renal hypoplasia in mice (Keller et al., 1994; Gruss and Walther, 1992), and humans (Sanyanusin et al., 1994). Collectively, these data imply that pax-2 regulates the transcription of genes required for the first stage of renal epithelial morphogenesis, and that continued expression of such genes may perturb later stages of renal differentiation, such as cell diversification and/or segmented tubule formation.

1.3.6.3 Cell adhesion and the extracellular matrix

Once the mesenchyme has become induced, many of its cells begin to adhere more tightly to one another. This is reflected in the expression patterns of the adhesion molecules and extracellular matrix. Uninduced mesenchyme expresses collagen I, III, and fibronectin (Ekblom, 1981). The neural cell adhesion molecule (NCAM) is expressed by the uninduced mesenchyme, with its expression being down regulated as the nephron matures (Klein et al., 1988; Lackie et al., 1990). Following induction, the mesenchymal cells begin their transition to polarised epithelia, losing the expression of interstitial collagens and fibronectin. Concurrently, they begin to express uvomorulin (E-cadherin), collagen type IV, and laminin A chain (Ekblom, 1981; Vestweber et al., 1985). Laminin is a large multidomain cruciform glycoprotein, and is a major constituent of basement membranes, with several isoforms known to exist. Uninduced metanephric mesenchyme expresses the B1 and B2 chains, whilst the A chain is expressed at the onset of epithelialisation. The addition of laminin A chain blocking antibodies to organ culture disrupts tubule formation by preventing polarisation of the
epithelial cells (Klein et al., 1988). Antibodies that bind to the distal part, or the long arm of the molecule, have been found to be most effective.

Integrins are the major family of molecules that serve as cell surface receptors for components of the extracellular matrix (ECM) (Kreidberg et al., 1996). They are heterodimeric cell surface receptors, composed of a single $\alpha$ and $\beta$ peptide subunit. The extracellular domains of integrins interact with the ECM, while some cytoplasmic domains interact with the cytoskeleton (Chen et al., 1995). Within an integrin subfamily, a single $\beta$ subunit is able to form heterodimers with several $\alpha$ subunits. The extracellular domain of the $\alpha$ subunit confers the binding specificity for the heterodimer, and the particular biological response to binding is determined by the $\alpha$ subunit cytoplasmic domain (Chan et al., 1992). Upon integrin binding to components of the ECM, signals are transduced into the cell. These signals control diverse cellular behaviours such as adhesion and cell migration (Clark and Brugge, 1995). $\alpha_3$ integrin is expressed during the development of many epithelial organs, including the metanephros (Kreidberg et al., 1996; Korhonen et al., 1991). $\alpha_3\beta_1$ has been shown to participate in homophilic (Sriramarao et al., 1993) and heterophilic (Symington et al., 1993) interactions with other integrins, suggesting it may play a role in cell-cell adhesion. In the $\alpha_3\beta_1$ null mutant mice, there is decreased branching of the medullary collecting ducts, with cysts developing within the proximal tubule epithelia and glomerular development also being affected (Kreidberg et al., 1996). The $\alpha_6$ integrin is also involved in tubulogenesis, since blocking antibodies to this integrin perturbs tubule formation (Sorokin et al., 1997). A further basement membrane glycoprotein, nidogen, is produced by mesenchymal cells and binds to laminin. This binding is crucial for the production of epithelial basement membranes formed during early nephrogenesis (Ekblom et al., 1994).

Both the epithelia and mesenchymal cells express sulphated glycosaminoglycans (GAGs), which are up regulated during nephron formation. Syndecan is a proteoglycan which acts as a receptor for interstitial matrix molecules and is a marker for the early mesenchyme-epithelial transition (Vainio et al., 1989). GAGs may function in the presentation of growth factors to high affinity receptors (Rapraeger et al., 1991). Blocking GAG activity prevents ureteric bud growth and branching, but has little effect on nephron development (Davies et al., 1995). However, branching of the ureteric bud can be restored by the addition of PMA (phorbol 12-myristate acetate), whilst proliferation can be restored by the addition of HGF. Together, this data suggests that growth and branching of the ureteric bud are distinct processes. Surprisingly, nephron formation in these cultures is normal, indicating that nephrogenesis is dependent on the presence, but not on the development, of the ureteric bud.
1.4 The development of the lung

In the mouse, lung development begins at E9.5 when bilateral epithelial lung buds sprout from the primitive gut endoderm and grow in a ventrocaudal direction, invading the mesenchyme of the splanic mesoderm (Figure 1.2). These are the rudiments of the two lungs, the left and right primary bronchioles. Patterning of the airways is subsequently accomplished by growth and repetitive branching, under the inductive control of the mesenchyme (Larson, 1993).

Figure 1.2 Partitioning of the foregut into the oesophagus and respiratory diverticulum

(A) Lateral view, E10. (B,C) Ventral views. Figure taken from Gilbert (1988).
There are five stages of lung development: embryonic, pseudoglandular, canalicular, saccular, and alveolar. The embryonic stage consists of the initial outgrowth of the lung buds and the initial three rounds of branching. This produces the primordia of the two lungs; the lung lobes, and the broncho-pulmonary segments. During the pseudoglandular stage (E10-16), the respiratory tree undergoes further branching, resulting in the formation of terminal bronchioles. By E14, the epithelium has differentiated into proximal (or bronchial) epithelium which is columnar, and distal (or respiratory) epithelium which is cuboidal. Each terminal lung bud divides into two more respiratory bronchioles during the canalicular stage (E16-17). During the saccular stage (E17-birth), the bronchioles further divide to form sac-like structures (the primitive alveoli). This process is accompanied by a thinning of the mesenchyme and an invagination of blood vessels. The final maturation of the alveoli then occurs during the alveolar stage. The mesenchyme eventually develops into the connective tissue, the pulmonary smooth muscle and the vasculature of the adult lung. In contrast to the adult kidney, where both the mesenchyme and epithelia contribute to the epithelial structures of the organ, the adult lung epithelial structures are derived from embryonic epithelia only.

In Drosophila, the trachea is formed by a process analogous to branching morphogenesis of the vertebrate lung (Bier et al., 1989), suggesting that branching morphogenesis is a primitive and highly conserved morphogenetic process. Branching of the Drosophila trachea is not sequential, but follows an iterative branching pattern (Samakovlis et al., 1996), producing proximal structures that differ greatly from distal structures.

1.4.1 Recombination studies

From early recombination studies by Grobstein, it is clear that the mesenchyme has an instructive inductive effect on epithelia (Grobstein, 1967). Recombination of the early lung bud epithelia with mesenchyme from distinct organs, supports growth and development of the epithelia. However, the lung buds do not develop into structures typical of the lung, but into structures typical of the organ from which the mesenchyme is derived (Deucher, 1975) (Figure 1.3). For example, if lung bud epithelium is recombined with stomach mesenchyme, it develops gastric glands, whilst if it is recombined with intestinal mesenchyme, it develops villi typical of the gastrointestinal tract. This supports the hypothesis that each mesenchyme has an instructive influence on the epithelium. An elegant series of experiments performed by Spooner and Wessels demonstrate that the mesenchyme surrounding the trachea and mainstream bronchi has a different regional identity to that surrounding the distal lung bud. Tracheal epithelia can be induced to branch by bronchiial mesenchyme, whereas the branching of bronchiial epithelia cannot be supported by tracheal mesenchyme (Spooners and Wessells, 1970; Wellicks,
Figure 1.3 Ability of presumptive lung epithelium to differentiate with respect to the source of the mesenchyme.


(A) Lung epithelium does not differentiate when cultured in the absence of mesenchymal cells. (B-F) Mesenchyme-specific differentiation of epithelium.

Figure taken from Gilbert (1988).
This regional specificity of induction is critical during the development of the digestive and respiratory systems. In the morphogenesis of the endodermal tubes, the epithelium is able to respond differently to the regional specific mesenchymes. This enables the digestive and respiratory tubes to develop within their appropriate positions. Comparisons of these studies with expression studies, reveals that these mesenchymal identifies are held within the spatial expression patterns of specific genes. Transcription factors, growth factors and their receptors, along with ECM molecules and their receptors, all play a part.

1.4.2 Molecular control of lung development

1.4.2.1 Cytokines and their receptors

Comparing the metanephros to the lung, one can see that several differences have been reported. For example, the receptor tyrosine kinase ros is expressed from the initial stages of metanephric development, and is down regulated in the adult, whereas in the lung, expression is not seen until E14, which then continues within the adult organ (Sonnenberg et al., 1991). Several members of the FGF family are known to effect both lung and metanephric development. However, close examination reveals that in the metanephros basic FGF (bFGF) induces condensation of the mesenchyme, whereas in the lung, acidic FGF (aFGF) is crucial for epithelial growth in mesenchymal free cultures (Nogawa and Ito, 1995; Perantoni et al., 1995). These studies demonstrate the importance of isolated epithelial and mesenchymal culture systems. Another FGF family member, keratinocyte growth factor (KGF or FGF7), is expressed in lung mesenchyme near positions of branching, with its receptor (KGFR) being expressed in the epithelium (Post et al., 1996). The mesenchymal-epithelial distribution of KGF and KGFR has also been documented during development of the metanephros and salivary gland (Robin et al., 1995). Mice harbouring null mutations in FGFR exhibit dramatically reduced bronchial branching (Peters et al., 1994). Lung development is also disrupted, in organ culture, by the addition of blocking antibodies or oligonucleotides to KGF or KGFR (Post et al., 1996). Interestingly, deletions of breathless, an FGFR gene, causes abnormal tracheal branch migration in Drosophila.

Other growth factor families can be shown to exhibit similar effects on both lung and metanephric development. Null mutations for the PDGFA and PDGFB genes cause a failure in development of the lung and kidney respectively (Nogucki et al., 1989; Leveen et al., 1994). PDGFB mutants have a complete absence of renal fibromuscular mesangial cells, while PDGFA mutants have a defect in their alveolar myofibroblasts, the source of septal elastin. Although PDGFA null mutants have no kidney phenotype, there is a degree of similarity between them and the PDGFB mutants. Mesangial cells, like alveolar myofibroblasts, express
α-smooth muscle actin and have a contractile phenotype. Both cell types act as an 'anchor' for an involuted epithelial sheet, with loss of cellular function leading to a loss of involution. This results in decrease of surface area and hence function (glomerular filtration or gaseous exchange). Thus, there is a clear analogy between the growth factor dependence, and developmental roles, for mesangial cells and alveolar myofibroblasts.

The platelet-derived growth factor (PDGF) scenario demonstrates that, within the diversity amongst organs, there are also similarities. Both PDGFA and PDGFB act to enhance development. TGFβ is a molecule that inhibits branching morphogenesis in both the lung (Heine et al., 1987; Serra et al., 1994; Zhou et al., 1996) and kidney (Pachnis et al., 1993; Rogers et al., 1993). It can therefore be presumed that TGFβ acts to control the final size of these adult organs.

Members of the hoxb family have been suggested to play a role in the spatial regionalisation of the developing lung (Bogue et al., 1994). One would expect this regionalisation to manifest itself at a higher level, in the spatially differential expression patterns of growth and morphogenic factors. This is indeed the case, as seen with members of the BMP family and sonic hedgehog (shh). BMP4, BMP7, and shh are expressed by the epithelium of the developing lung. Shh appears more uniformly across the epithelia, with only a slight increase at the distal tips. BMP4 and 7 show a much more polarised expression pattern, with the highest levels being at the developing tips. BMP4 is also expressed in the mesenchyme around the distal tips (Bellusci et al., 1996).

### 1.4.2.2 Transcription factors

Based on the expression patterns of the *hox* genes, it is probable that the expression of a specific combination of these genes is involved in the molecular mechanisms responsible for the budding of the lung from the tracheal oesophageal portion of the fore gut. There are fifteen hox genes expressed in the developing lung (Bogue et al., 1994). Hoxb cluster genes are expressed in overlapping spatial domains during lung development. 3' cluster genes are expressed more anteriorly in the branchial arches and foregut, with 5' genes being expressed more posteriorly. Within the mesenchyme, expression of these genes is not collinear but forms two general patterns: expression in both proximal and distal lung (hoxb-3 and b-4) and expression in the distal lung only (hoxb-2 and hoxb-5) (Bogue et al., 1996). Thus, *hox* genes may be involved in specifying the spatial domains within the lung mesenchyme.

It has already been mentioned that pax-2 is a transcription factor expressed during metanephrogenesis, with loss or misexpression of this gene being detrimental to normal renal development. It is interesting to note that there are no reports of pax-2 being expressed in the
developing lung, suggesting that it may be part of the mesenchymal specificity originally postulated by Grobstein (Grobstein 1967).

1.4.2.3 ECM and their receptors

One of the ways in which mesenchymal cells exert their influence on branch formation is by stabilising the basement membrane in clefts, or destabilising it enzymatically where branches are to be initiated (Bemfield et al., 1997). Branching morphogenesis of the lung and metanephros occurs through a process of budding, and hence one might expect to see many similarities in the expression of the ECM and its receptors.

As previously discussed, within the metanephros, laminin is involved in the mesenchymal to epithelial conversion of the condensates; blocking antibodies to laminin disrupting this process (Klein et al., 1988). Conversely, within the lung, blocking the function of laminin disrupts the growth and branching morphogenesis of the lung epithelium (Shuger et al., 1991). The role that laminin plays during lung branching morphogenesis is highlighted by its localisation to the epithelial-mesenchymal interface of the terminal lobes (Thomas and Dziadek, 1994). α3 integrin is expressed during lung development, the protein being localised to the basolateral region of the cells (Kreidberg et al., 1996; Mette et al., 1993). In α3β1 null mutant mice, along with kidney malformations, the branching of the bronchi is also decreased, thus indicating a role for integrin receptors in both basement organisation and branching morphogenesis of these two organs (Kreidberg et al., 1996).

From the above discussion, it can be seen that the kidney and lung develop in an apparently similar manner, and this is reflected in many similarities in the molecular control of their growth and development. However, there are some important differences, which is why this study includes investigations into the metanephros, lung, and salivary gland development.

1.5 The development of the submandibular salivary gland

Three pairs of salivary glands develop in mice and humans: the parotid, submandibular, and sublingual (Larson, 1993). The salivary gland is first detectable late on E12 of mouse gestation. The salivary gland primordium results from an invagination of the oral epithelial sheet into the mandibular mesenchyme. Initially, it consists of a stalk (primary duct) and a bulbous lobe, in a similar fashion to the beginnings of metanephric and lung development. However, unlike the ureteric bud and lung epithelium, the initial epithelial bud is not a sheet, but a mass of cells. Only after several rounds of branching, governed by the inductive interactions between the epithelia and the mesenchyme, does the lumen formation occur. From this time onwards, the epithelium takes on a cell sheet arrangement. The oral
epithelium which gives rise to the salivary gland is partially differentiated. The epithelial cell mass loses markers of differentiation, but as lumen formation occurs, and the epithelial cells once again take on the morphology of a cell sheet, these markers are re-expressed (Heida et al., 1996). Hence, during the early formation of the salivary gland, the epithelium goes through a round of dedifferentiation followed by epithelialisation. Unlike the other two organs described, the salivary gland branches through a process of clefting rather than budding (Figure 1.4), but as with the lung, the mesenchyme does not undergo a mesenchymal to epithelial transition.

1.5.1 Molecular control of clefting in the developing salivary gland

Spooner et al. demonstrated that epithelial proliferation is not required for early cleft formation (Spooner et al., 1989). However, it was later demonstrated that epithelial proliferation is indispensable for complete branching morphogenesis (Nogawa and Takahashi, 1991). Branch point formation requires microtubules, microfilaments, and an intact basal lamina. The mesenchyme subtly controls the developmental changes occurring in the organ, partly through highly localised deposition and remodelling of the ECM. Cleft formation has been conclusively linked to the contractile behaviour of microfilaments at the basal and apical sides of the epithelial cells. The mesenchymal cells, together with collagen fibres, exert tractional forces on the epithelial cell surfaces to result in the epithelial shape change required for cleft formation (Nakanishi and Ishii, 1989; Fukuda et al., 1988). Collagenase inhibitor markedly enhances cleft formation, whereas the addition of collagenase inhibits it (Nakanishi et al., 1986). Abundant collagen-like fibrils are seen in the collagenase inhibitor treated organs, showing that the accumulation of collagen is one of the motive forces which ensure cleft formation.

Growth and morphogenesis of isolated salivary gland epithelia can be supported in three dimensional cultures when grown in a Matrigel™ matrix with exogenous EGF or TGFα (Nogawa and Takahashi, 1991). TGFα binds the EGF receptor, and is therefore thought to work through the same intracellular mechanism. The culture of epithelium alone demonstrates that it can branch without the influences of the mesenchyme, including its tractional forces and basement membrane degrading enzymes. Interestingly, different branch patterns are produced by the addition of different concentrations of EGF. This suggests a possible dose dependent mechanism through which the mesenchyme can exert its instructive influence upon the epithelium.
(a) Proteoglycans produce a high net negative charge in certain areas of the basal lamina and bind large amounts of calcium (Ca). The large arrow indicates site of future branch-point formation. Growth factors (GF) complete with calcium ions for binding sites on the proteoglycans. (b) Calcium ions (Ca\(^{2+}\)) displaced from the basal lamina enter the cells. The influx of calcium ions stimulates microfilament contraction with narrows the basal ends of these cells. Co-ordinated contraction occurring across many cells results in cleft formation. (c) The forming cleft continues to deepen. The speed of this process throws the basal lamina into folds at the base of the cleft. Meanwhile, localised degradation of proteoglycans by mesenchymal enzymes at the lobule tips releases the growth factors. These can then act locally on the epithelial cells to stimulate cell division and branch outgrowth. Proteoglycans are quickly resynthesized and again produce a high net negative charge at the distal lobules. (d) New branch-points will be initiated at the sites indicated by the large arrows.

Figure taken from Hardman (1989).
1.6.1 Discovery of ret

The human \textit{RET} gene (REarranged during Transfection), as its acronym suggests, was first defined as a dominant oncogene in a classical DNA transformation assay. The novel transforming gene originated from an artefactual rearrangement that occurred in vitro (Takahashi et al., 1985). It was subsequently shown to be a receptor tyrosine kinase (Takahashi et al., 1988), although, until recently the ligand for ret remained unknown. In 1996 the ligand was demonstrated to be GDNF (Trupp et al., 1996; Durbec et al., 1996). The transmembrane ret glycoprotein is expressed in two isoforms, 1072 and 1114 amino acid. These differ from each other in their 9 and 51 carboxy-terminal amino acids, which are generated by the alternative splicing of the last two exons (Tahira et al., 1990).

1.6.2 Structure of ret

The \textit{ret} gene encodes a member of the receptor tyrosine kinase superfamily. Members of this family include growth factor receptors, such as the EGF receptor, the high affinity nerve growth factor receptor (trk), and the HGF receptor, met. Each of these family members has a large extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain that has protein tyrosine kinase activity. These receptors are believed to play fundamental roles in cell-cell signalling through their interactions with specific ligands, the binding of which promotes receptor dimerisation and activation of the tyrosine kinase domain (Ullrich and Schlessinger, 1990). The activated receptors phosphorylate specific intracellular substrates that, in turn, regulate cell growth and differentiation. Thus, receptor tyrosine kinases are vital mediators of cell-cell communication, a process that is essential for both the correct induction of embryonic tissue during development and for growth regulation.

1.6.3 Sequence similarity to the cadherin superfamily

The extracellular domain of ret shows a unique structural feature, the presence of sequences similar to cadherin repeats (Iwamoto et al., 1993). Cadherins are transmembrane proteins that mediate homophilic, calcium-dependent cell-cell adhesion (Takeichi, 1991), a process important to differentiation, morphogenesis, and tumour suppression. The homologous domains contain the calcium-binding motifs necessary for homophilic adhesion, and for the resistance to proteolysis. Remarkably, the repetitive regions identified in ret are not only homologous in position and sequence to the cadherin calcium-binding sites, but also coincide exactly with the best conserved hallmark residues of the cadherin family. Cells transfected with ret, however, do not demonstrate homophilic binding, or increased cellular aggregation (Takahashi et al., 1993).
1.6.4 The involvement of RET in cancer

Mutations in RET are associated with different cancer diseases; papillary thyroid carcinoma (PTC) (Herrmann, 1996), familial medullary thyroid carcinoma (FMTC) (Gagel, 1995), multiple endocrine neoplasia type 2A (MEN 2A), and MEN 2B (Gagel, 1995). The oncogenic activation of RET is due to genomic rearrangements, in which the extracellular domain is replaced by amino-terminal protein fragments from various other genes. Such tumour-specific rearrangements, usually associated with abnormally sized mRNA, are frequently seen in papillary thyroid carcinomas, tumours of thyroid epithelia which do not normally express RET. High-level expression of normal sized RET mRNA is found in medullary thyroid carcinomas (calcitonin-secreting, C-cell tumours), and in phaeochromocytomas (adrenal chromaffin cell tumours). Families with these cancers can be classified, according to their phenotype, into three different categories; FMTC, MEN 2A, and MEN 2B. In MEN 2A, medullary thyroid carcinoma is often accompanied by phaeochromocytoma and parathyroid hyperplasia (Mulligan et al., 1993). MEN 2B has all the features of MEN 2A, but has an early tumour onset and is characterised by ganglioneuromas (Santoro et al., 1995). All of these cancer syndromes are associated with highly specific, heterozygous, amino acid substitutions, suggesting that a single dominant hit at the RET locus is sufficient to initiate tumours. In view of the postulated role of RET in renal development, it is interesting to note that patients with these tumours do not have renal carcinomas.

1.6.5 The involvement of RET in Hirschsprung's disease

Hirschsprung's disease is a congenital disorder of the enteric nervous system affecting around 1 in 6,000 people. It is characterised by the absence of parasympathetic intrinsic ganglion cells in the submucosal and myenteric plexuses of the hindgut. The result is severe constipation, with abdominal distension and intestinal obstruction appearing in the first year of life. Genetic mutations associated with Hirschsprung's patients include heterozygous mutations in the RET gene (Carломagno et al., 1996). These mutations are deletions and loss-of-function changes, such as premature stop codons (Pasini et al., 1995). Again, these patients very rarely present with any renal malformation.

1.6.6 Expression of ret in mouse and rat

There have been many reports concerning the expression of ret in the developing mouse embryo, some of which have been conflicting and inconclusive (Tahira et al., 1988; Avantaggiato et al., 1994). However, a detailed study by Pachnis et al. does define a clear pattern of ret expression in the developing nervous and excretory system of the mouse (Pachnis et al., 1993). By the use of Northern blot analysis on adult tissues, four transcripts can be
detected, migrating at 7.0, 6.0, 4.5, and 3.9 kb, the 4.5 kb transcript being the most abundant. The adult brain and salivary gland show strong expression (Pachnis et al., 1993), the heart, spleen, and c-cells of the thyroid show low expression (Avantaggiato et al., 1994), whilst the kidney, liver, ovary, thymus, lung, adrenal medulla, and testis show no expression (Pachnis et al., 1993).

1.6.6.1 Expression of ret during organogenesis of the pro, meso, and metanephros

From E9, the transient embryonic structures of the pronephros, and subsequently the mesonephros are present within the embryo. Ret expression is detected within the nephrotomes and Wolffian ducts of these structures (Pachnis et al., 1993). During metanephric development (from E11.5 onwards), only the epithelial cells of the branching ureteric bud express ret. At later stages, the nephrogenic interactions occurring in the kidney are confined to the outer nephrogenic zone, and it is here that ret expression can still be detected within the tips of the ureteric bud (Pachnis et al., 1993). While it has been reported that the ret protein can be detected in the metanephric mesenchyme and its derivatives (Liu et al., 1996), these data are thought to be controversial and disagree with findings in many other studies, including the current one (Pachnis et al., 1993; Tsuzuki et al., 1995; Towers et al., 1997).

1.6.6.2 Expression of ret during organogenesis of the liver

A scattered in situ hybridisation signal has been reported within the developing liver at E12.5 and E14.5. This was confirmed by the use of Northern analysis, although the data are not shown (Avantaggiato et al., 1994). However, these findings contradict the results of this study, as discussed in Chapter 3.

1.6.6.3 Expression of ret in the developing sensory nervous system

Ret is expressed in both the mesenchymal (early emerging) and neurogenic (late emerging) components of neural crest cells migrating from rhombomere 4 (r4). Expression is later detected within the post mitotic derivatives of these neural crest cells, the anlage of the facioacoustic ganglion complex. Also at this stage, expression is detected in a well defined domain of cranial surface ectoderm and pharyngeal endoderm associated with the posterior, but not anterior, branchial arches. This area also includes the epibranchial placodes, which give rise to the sensory neurones of the inferior ganglia within the IXth and Xth cranial nerve ganglion complexes. As development progresses, ret expression is seen in all cranial ganglia, irrespective of the contributing placode or neural crest origin (Pachnis et al., 1993). Tsuzuki et al. demonstrated a similar pattern of protein expression in the IXth and Xth cranial ganglia (Tsuzuki et al., 1995).
Ret is also expressed by the neural crest cells of the trunk. Areas of the somites, in which the trunk neural crest cells first migrate and subsequently condense to form the dorsal root ganglia (DRG) between the dorsal neural tube and surface ectoderm, show weak expression. Subsequently, the DRGs have an intense punctate signal within the ganglion itself, but not in the emerging peripheral spinal nerve (Pachnis et al., 1993). Thus, ret is expressed in a subset of the DRG neurones and is absent from the glial cells, as is also found with the protein expression (Tsuzuki et al., 1995).

In the E17.5 mouse embryo, Avantaggiato demonstrated a faint signal restricted to a few mesenchymal cells surrounding the olfactory epithelium (Avantaggiato et al., 1994).

### 1.6.6.4 Expression of ret in the developing central nervous system (CNS)

Within the CNS, expression of ret is restricted to the ventrolateral compartment of the spinal cord, where the motor neurones differentiate. This expression can be detected up to E14.5. The ventral pattern of expression extends along the entire anterior-posterior axis of the developing spinal cord, and continues anteriorly into groups of motor neurones in the hindbrain (Pachnis et al., 1993).

Sections through the eye of 13.5 embryo show signal to be restricted to the innermost layers of the neuroretina, where the first postmitotic neurones are born. In the neonatal mouse, all classes of neurones and glial cells of the retina have developed. At this stage, expression is localised to certain layers of the retina, containing the ganglion, amacrine and horizontal cells. No expression is detected in the layers that contain the bulk of the bipolar cells and the photoreceptors (Pachnis et al., 1993; Avantaggiato et al., 1994).

### 1.6.6.5 Expression of ret in the developing peripheral nervous system (PNS)

Ret is expressed concomitantly in the anlage of the sympathetic ganglia, and in the precursors of the enteric nervous system (ENS). Between E10 and E10.5, a stream of positive cells are seen emerging from the posterior branchial arches, migrating towards the foregut. Subsequently, positive cells are seen at more caudal levels of the gut, where they now express the protein (Tsuzuki et al., 1995). At later stages, once the rostrocaudal migration of the ENS precursors is complete, strong expression is detected in the myenteric plexus along the entire axis of the gut, i.e. the majority of the migrating precursors and postmigratory cells of the ENS (Pachnis et al., 1993). The expression of ret in the migratory pathways of the presumptive enteric neuroblasts suggests that it plays a role in the migration of the vagal crest during the development of the ENS. This theory has since been strengthened by the phenotype of the ret
null mutant mice, which lacks neurones of the myenteric plexus from the small and large intestine (Schuchardt et al., 1994).

Expression is also seen within cells of the PNS by the dorsal aorta. It is here that the trunk neural crest coalesces to form the sympathetic ganglia. Expression is maintained in these ganglia at later stages (Pachnis et al., 1993).

1.6.7 Expression of ret during avian development

There have been two published studies on the expression of ret in the avian embryo (Schuchardt et al., 1996; Robertson and Mason, 1995). Data from these two reports are discussed below and compared to that reported for the mouse. Similarities in the sequence and expression patterns between the mammalian and avian species are likely to be of importance, since they have been conserved during evolution.

As discussed above, the mouse (Iwamoto et al., 1993) and human (Kuma et al., 1993) ret extracellular domains contain a 110 amino acid region with similarity to a repeat found in the extracellular domains of cadherins. This cadherin-like domain has also been found in the avian sequence. Its sequence similarity to other members of the cadherin family is relatively low, suggesting that ret is a very distant family member. Interestingly, the putative calcium-binding sites are conserved in the ret proteins from all species, which supports the hypothesis that calcium may be involved in the binding of ret to its ligand (Kuma et al., 1993). In addition to the conserved motifs, the avian ret gene also encodes two protein isoforms, which differ from each other only in their carboxy-terminal sequences.

Northern blot analysis of adult tissues reveals a similar distribution of ret expression in the chicken (Schuchardt et al., 1996) to that seen in the mouse (Pachnis et al., 1993) and human (Tahira et al., 1990), the only exception to this being a high level of expression in the chicken testes. This suggests a high level of conservation between the adult expression patterns in these species.

With the use of in situ hybridisation, chicken ret can been shown to be localised to the developing renal system, as described for the mouse (Pachnis et al., 1993; Tsuzuki et al., 1995). Ret is also detected in the Wolffian duct prior to ureteric bud outgrowth, and subsequently in the growing and branching ureteric bud tips at later stages (Schuchardt et al., 1996; Robertson and Mason, 1995). As described for the mouse, ret expression is also detected in the neural crest cells that form the ENS, DRGs, sympathetic ganglia of the PNS, and the ventral neural tube of the CNS.

Several differences have also been reported between the avian and mouse embryos. In the chicken, ret expression is observed in the cranial mesenchymal cells as they migrate into the branchial arches, cells in which expression is not seen in the mouse (Pachnis et al., 1993).
Also, both the facioacoustic and trigeminal ganglia express ret early in chicken development, whereas in the mouse, the trigeminal ganglion does not begin to express ret until E13.5. In addition, chicken ret is found to be expressed along the ventral roots (probably neural crest derived glial cells), but it not detected in the corresponding mouse cells.

1.6.8 Expression in *Drosophila*

In all vertebrate species studied so far, four RNA transcripts have been observed. In *Drosophila*, however, only a 4.5kb RNA species (the most abundant in vertebrates) is detected in the larval and pupal stages (Sugaya et al., 1994).

In early embryos, Dret is transiently expressed in a subpopulation of neuroblasts. In later embryos, it is detected in a subpopulation of differentiating CNS cells, including ganglial cells of the supraoesophageal. Expression is also detected in the lateral region, an area related to the developing PNS. This expression is transient, and limited to certain cell populations within the CNS and PNS (Sugaya et al., 1994).

In summary, it can be seen that the expression profile of *Drosophila* ret shows striking similarity to that reported for vertebrate species, suggesting that both *Drosophila* and vertebrate ret are involved in similar developmental processes.

1.6.9 Transgenic null mutant mice for ret

Transgenic mice were derived by inserting a neo\(^R\) gene into the ret gene, simultaneously deleting 0.8 kb of DNA, including the codon for an invariant lysine required for kinase activity. Mice heterozygous for the mutant gene appeared phenotypically normal. Further breeding of these mice resulted in the birth of homozygous mutant animals, which died 16-24 hours after birth. The most striking defect in these mice was either absent, or severely dysplastic kidney rudiments, along with the absence of ganglia from the gastrointestinal tract (Schuchardt et al., 1994).

1.6.9.1 Defects in metanephros development

Some homozygous mice contain blind-ending ureters with no renal tissue, while others display a complete absence of the ureter and kidney, either unilateral or bilateral (Schuchardt et al., 1994). The metanephric rudiments show a great variety of defects; reduced numbers of recognisable nephric elements (including proximal and distal tubules, glomeruli and vessels), no recognisable metanephric regions, reduced branches of the ureteric bud without the formation of mature collecting ducts, and large regions of undifferentiated mesenchyme.

Recombination studies between wild type or mutant ureteric buds and wild type or mutant metanephric mesenchyme, demonstrate that the primary lesion is intrinsic to the ureteric
bud. Wild type spinal cord can induce mutant renal mesenchyme to differentiate as normal, whereas the mutant bud fails to respond to wild type mesenchyme (Schuchardt et al., 1996). The authors suggest that ret is the receptor for a mesenchymally derived factor.

1.6.9.2 Defects in enteric nervous system

The second abnormality in all homozygous mutant pups, is a failure of milk to progress from the stomach into the intestine, indicating a defect in gastrointestinal peristalsis. This abnormality is due to an absence of myenteric plexus neurones from the small and large intestine, the oesophagus and the stomach. This demonstrates a critical role for ret in the establishment of the enteric nervous system (Schuchardt et al., 1994). Inactivation of ret prevents the successful colonisation of the midgut and hindgut by enteric neuroblasts, although the foregut is only partially affected (Durbec et al., 1996). The neural crest precursors of the midgut, hindgut and superior cervical ganglia, share a common origin from the postotic hindbrain. In contrast, the foregut neurones are likely to be derived, along with the remainder of the sympathetic chain, from trunk level neural crest. This explains the phenotype of the null mutant mice, in that two genetically distinct lineages contribute to the formation of the enteric nervous system.

1.6.10 The ligand for ret is Glial cell line-derived neurotrophic factor

For many years the ligand for ret remained an enigma, although it was predicted to be a mesenchymally-derived secreted protein. In 1996, two papers published simultaneously in Nature, presented work demonstrating that the ligand for ret was the neurotrophic factor, GDNF (Trupp et al., 1996; Durbec et al., 1996).

1.7 Glial cell line-derived neurotrophic factor

1.7.1 Discovery and initial characterisation

GDNF was first isolated from the rat glial cell line B49 (Lin et al. 1994). It is a glycosylated, disulfide-bonded homodimer and has been included into the TGF-β superfamily due to a TGF-β-like precursor structure, dimeric subunit composition, and the fact that it contains a set of cysteines typical of the family. The cysteines form three disulfide bonds, interlocked into a tight structure known as a 'cysteine knot' (Sun and Davies, 1995). TGF-β family members are thought to signal through heterodimeric complexes of high affinity receptor serine-threonine kinases (type 1 and 2 receptors), and low affinity betaglycan receptors (type 3 receptor) (Wrana et al., 1992). The fact that GDNF binds to a receptor tyrosine kinase is a
breach of family tradition, and may reflect GDNF's status as the most distant TGF-β relative known to date. Furthermore, the identification of a receptor tyrosine kinase as the signal transducing component of a TGF-β signalling complex, has profound implications to the receptor binding of these ligands, the implication being that they may possibly be more promiscuous than is currently believed. However, there is some debate as to whether GDNF should be included in the TGF-β superfamily, since versions of the 'cysteine knot' are present in ligands belonging to other families. For example, nerve growth factor (NGF) and platelet-derived growth factor (PDGF), both of which signal through typical receptor tyrosine kinases, along with human chorionic gonadotrophin, which is known to signal through a G-protein-coupled receptor, all contain 'cysteine knots' (Sun and Davies, 1995).

### 1.7.2 The ménage à trois of GDNF receptor binding

The finding that a TGF-β family member signals through a receptor tyrosine kinase is remarkable enough. However, the situation is further complicated by two papers by Jing et al. (1996) and Treanor et al. (1996) that present data suggesting that the binding of GDNF to ret involves the presence of a third molecule, GDNF receptor alpha (GDNFRα) (Jing et al., 1996; Treanor et al., 1996). GDNFRα is a novel glycosylphosphatidylinositol-linked cell surface receptor which binds GDNF specifically and with high affinity, but lacks a cytoplasmic domain capable of mediating transmembrane signalling. GDNFRα is a necessary component of the active GDNF receptor complex. Binding and cross-linking studies demonstrate that the association of GDNF with ret is mediated by GDNFRα, resulting in the activation of the ret tyrosine kinase (Jing et al., 1996; Treanor et al., 1996). GDNF is unable to stimulate ret autophosphorylation in cells that do not express GDNFRα, or in cells where GDNFRα has been removed, unless soluble GDNFRα is added to the culture medium (Jing et al., 1996).

Of further interest is that the concentration of GDNF required to trigger a biological effect varies widely among different cell types, suggesting the existence of both high and low affinity GDNFRα molecules. Jing et al. (1996) report high affinity binding of GDNF at similar levels to that presented by Lin et al. (1994), whereas Trupp et al. demonstrate very low affinity binding to chick sympathetic neurones. There is no evidence that GDNFRα is involved in this low affinity binding. It may be due to a weak direct interaction between GDNF and ret, although it is more likely to suggest the existence of low affinity GDNFRα isoforms.

### 1.7.3 GDNF as a survival factor

Purified native GDNF is a potent, and relatively specific, neurotrophic factor for dopaminergic neurones (Lin et al., 1994). GDNF selectively enhances the survival and development of these neurones, increasing the number and size of cells surviving in culture, as
well as dopamine uptake, and extension of neurites. GDNF is involved in one of the cellular inter-relationships between neurones and glia. This distinguishes GDNF from other factors known to affect midbrain dopaminergic neurones, such as IGF, EGF, and TGFα, which work indirectly through the glia (Knusel et al., 1990; Engele and Bohn, 1991; Alexi and Hefti, 1993). GDNF is not only active in vitro, but is also effective in vivo (Tomac et al., 1995). It has also been shown to have broader actions, including neurotrophic effects on developing motor neurones (Henderson et al., 1994). However, the increased number of surviving neurones has not been linked to an increase in proliferation, as assessed by BrdU incorporation (Krieglstein et al., 1995).

1.7.4 Expression of GDNF in the mouse embryo

To date, there has been only one report concerned with the expression pattern of GDNF, by in situ hybridisation, in the developing mouse (Hellmich et al., 1996). The results from this paper are discussed below and compared to the expression of ret.

1.7.4.1 Expression of GDNF during mouse organogenesis

Non-neuronal GDNF expression is found in many organs that rely on interactions between epithelia and mesenchyme for their development. However, within these developing organs, GDNF is seen only in the mesenchymal component.

GDNF expression can be detected as early as E11.5 within the presumptive metanephric mesenchyme, but not in the ureteric bud. As development progresses, the ureteric bud branches, and is surrounded by condensing mesenchyme. At all times during nephrogenesis, GDNF transcripts are restricted to the undifferentiated mesenchyme adjacent to the condensing mesenchyme. This pattern is maintained through development to the latest reported stage, at E15.5. This observation correlates perfectly with the hypothesis that a mesenchymally derived ligand for ret has an inductive effect on the ureteric bud epithelia, guiding development of the metanephros.

At early stages of enteric development, the gut consists of a simple, elongating, epithelial tube. At this early stage, GDNF expression is seen in the mesodermal mesenchyme surrounding the gut endoderm. Later on in development, expression is observed along the entire length of the gastrointestinal tract, from the oesophagus to the rectum. During this period, the gut is surrounded by a layer of loose mesenchymal cells, which ultimately contribute to the formation of the connective tissue and smooth muscle. GDNF is primarily expressed over the outer mesenchymal layer and during later stages, within the outer smooth muscle layer. Ret positive ganglioblasts migrate rostrocaudally inside the gut wall mesenchyme, and eventually coalesce to form the enteric ganglia along the entire length of the
gut (Durbec et al., 1996). The survival of these cells is disrupted in ret null mutant mice (Schuchardt et al., 1994). GDNF is transcribed along the length of the gut where it is postulated to aid the survival, or proliferation of the ganglioblasts.

Reciprocal interactions between epithelial and mesenchymal tissues are also important in tooth morphogenesis. The tooth primordia can be detected for the first time between E13 and E14, and at this stage, GDNF is expressed in the tooth bud mesenchyme, suggesting an involvement in its development. GDNF is expressed in other mesenchymal tissue, including that underlying the tongue, and there is also strong expression in the connective tissue surrounding the vibrissae papillae. In addition, GDNF transcripts can be detected in the developing testes. Only two types of cells are identifiable in the testes cord by E15.5, GDNF being confined to the precursors of the Sertoli cells, not in the interstitium. Based on its spatial and temporal expression pattern in early embryogenesis, GDNF is ideally located to bind to ret and to be one of the many factors involved in epithelial-mesenchymal interactions.

1.7.4.2 Expression of GDNF in the developing limbs

GDNF is expressed very strongly throughout the mesenchymal tissues of the hind limb buds. Lindsay suggests that GDNF may have a target-derived role in motor neurone development (Lindsay, 1995). By E15.5, the developing digits have entirely separated, and GDNF can be detected in the mesenchymal connective tissue surrounding the cartilaginous components. It can also be detected in other mesenchymal tissues surrounding cartilage primordia, such as in the pericartilage mesenchyme of the femur and costae (Hellmich et al., 1996). Ret is not currently reported to be expressed within the developing limb, suggesting that GDNF may bind to receptors other than ret. However, this study demonstrates that a small population of ret positive cells can be detected within this structure.

1.7.4.3 Expression of GDNF in the developing central nervous system

At E9, GDNF transcripts are restricted to the anterior neuroectoderm, these being more widespread in the neuroepithelial wall of the prospective forebrain and midbrain. Expression is ultimately seen along the entire anterior-posterior axis of the CNS, with transcripts being enriched within the dorsal part of the neural tube. To date, GDNF has not been detected within the ventral midbrain (where dopaminergic cell bodies reside), or in dopaminergic target areas such as the ganglionic eminence, cortex, or olfactory bulb. However, within this project, the expression domains of GDNF in the developing mouse have been reassessed and expanded to include some of these areas.
Correlating to the expression of ret within the anterior portion of chicken somites (Robertson and Mason, 1995), GDNF expression is detected in the dorsolateral portion of mouse somites.

1.7.4.4 Expression of GDNF in the developing sensory nervous system

GDNF expression is found in the neuroepithelial wall of all brain regions, including the optic vesicles, the otic vesicles along with the first and second branchial arches. This is in contrast to the reported expression of ret, which is only detected in the more posterior arches (Pachnis et al., 1993). At E14.5, signal is seen in several mesenchymal components of the developing head, including the ear (around the acoustic epithelium), the nose, and the eye (the lens and optic cup, but not the retina). As before, this expression pattern closely matches that of ret (Pachnis et al., 1993), suggesting a role for the ret-GDNF signalling pathway in the development of these organs.

1.7.5 Transgenic null mutant mice for GDNF

Strains of mice carrying germline null mutations in the GDNF locus, were generated by blastocyst injection of recombinant embryonic stem cells (Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996). Mating of heterozygous mice results in the expected Mendelian ratio of wild type, heterozygous, and homozygous animals, thus indicating that GDNF expression is not absolutely required for embryonic development. However, all homozygous null mutant mice die within the first day of life. On closer inspection, it is found that the GDNF null mutant mice phenotype is very similar to the reported phenotype of the ret null mutant mice (Schuchardt et al., 1994).

Homozygous GDNF null mutant mice display renal agenesis or severe renal dysgenesis. However, unlike the ret mutants, whose heterozygous littermates are phenotypically normal, the GDNF heterozygotes display renal abnormalities. When analysed macroscopically, 35% of heterozygous mice between 3 and 5 months of age display a wide range of renal defects, including unilateral small kidneys with cortical cysts or rough surface (23%), and unilateral kidney agenesis or severe bilateral kidney dysgenesis (12%). Microscopic analysis reveals that metanephric development is halted, although a morphologically distinct blastema is present, confirmed by pax-2 staining at E12, even in the absence of ureteric bud outgrowth (Pichel et al., 1996).

Organ cultures of the heterozygous mutant urogenital region reveal a significant reduction in the growing arborisation of the renal collecting duct system, caused by either a delay in, or a complete absence of ureteric bud branching. Mutant ureteric buds can be caused
to grow and branch, in culture, by the implantation of agarose beads soaked in GDNF (Pichel et al., 1996).

The second abnormality found in the homozygous null mutant animals is the presence of milk in the oesophagus, and reduced progression of food into the gastrointestinal tract. This suggests a defect in peristalsis similar to that seen in the ret null mutants. This defect is due to the absence of neurones from the myenteric plexus within the small and large intestines, although a few neurones are present in the stomach. These results suggest that GDNF may be an early survival factor for neurones of the enteric nervous system (Moore et al., 1996).

1.7.6 The involvement of GDNF in congenital human disease

Mutations in RET have been linked to several human congenital diseases: PTC, MEN2A, MEN2B, FMTC, and Hirschsprung's. The discovery that GDNF is the ligand for ret has prompted the search for GDNF mutations in patients suffering with these diseases. To date, heterozygous germline mutations in GDNF have only been associated with a single Hirschsprung's patient, who presented with a short segment Hirschsprung's as well as a malrotation of the gut (Angrist et al., 1996). There were, however, no renal abnormalities. This patient also had a single base pair substitution in the RET gene, leaving the possibility that this was a polymorphism not involved in the disease phenotype.

1.7.7 Expression of GDNF in the mouse

Treanor et al. report the preliminary expression data for GDNF (Treanor et al., 1996). They demonstrate that, in the mouse, GDNF is found in many GDNF-responsive regions. Consistent with the phenotype of the ret and GDNF null mutant mice, GDNF is expressed in the developing ureteric bud. In the gut, expression is detected between the inner circular and outer longitudinal smooth muscle, adjacent to, and possibly within, the ENS. This coincides with the expression pattern of GDNF. Expression is also detected in the ventral midbrain (dopaminergic neurones), ventral spinal cord (spinal motor neurones) and in subpopulations of GDNF dependent DRG neurones. In addition, GDNFRα transcripts are seen in the outer layer of the tectum, choroid plexus, cerebellar primordium, olfactory epithelium, whisker pads, genital tubercle, urogenital sinus, testes, intervertebral discs and trachea, although much of these data are not shown in the article.

1.8 Experimental strategy
The aim of this project is to analyse the expression pattern and potential role of the ret signalling pathway during branching morphogenesis in the mouse embryo. This originally involved the characterisation of ret's function but with the discovery of its ligand in 1996 the scope of this project was expanded to cover ret, GDNFRa, and GDNF. This report contains four major sections, as outlined below.

1a) An initial investigation is undertaken into the expression windows of the three genes in developing mouse organs. RT-PCR is employed for this purpose, due to the minute quantities of RNA available from the embryonic tissues under investigation.

1b) To examine the definitive expression patterns of the genes, and to localise the expression to particular cell populations, in situ hybridisation is performed using dig-labelled antisense riboprobes.

2) Both ret and GDNF have been implicated in the early stages of nephrogenesis, but it is unclear whether this signalling pathway is involved in the development of other epithelial organs. For this reason, the metanephros, lung, and submandibular salivary gland are studied in organ culture. After growth in culture, the organs are stained with a laminin antibody, which binds to epithelial basement membrane. The organs are then visualised using the confocal microscope which allows the quantification of branch tips and developing nephrons.

3) The development of the metanephros relies upon the inductive interactions of both the epithelial and mesenchymal cell lineages. A lesion in the development of one affects the development of the other. For this reason ret is stably transfected into clonal cell lines of mesenchymal and epithelial origin. The function of GDNF induced ret activation can then be assayed without the confounding factors of the other cell types.

4) Organogenesis is a complex and intriguing developmental phenomenon. In vitro models, using clonal cell lines, are a useful tool to help dissect out the molecular mechanisms involved in the great orchestrations of these tissues. For this reason an in vitro, three dimensional, model of mesenchymal condensation is described. This system can be used in the future to study the roles of other genes, in nephrogenesis.
2. Methods and materials

2.1 DNA protocols

2.1.1 Agarose gels

0.8\% - 2\% gels were used depending on the size of DNA to be separated, smaller pieces of DNA being run on a higher percentage gel.

1) The agarose was dissolved in 50 ml 1 X TAE by gently heating in a microwave oven until there was full dissolution of the powdered agarose.

2) The solution was allowed to cool until it could be handled. 1\mu l of a 10 mg ml\(^{-1}\) ethidium bromide (EtBr) solution was added and the gel poured into the casting tray. Combs were inserted and the gel left for one hour to set.

3) When set, the combs were removed along with the casting tape and the gel was placed in the electrophoresis tank and covered with 1 X TAE.

4) Samples, along with a molecular weight marker, were mixed with a 10 X loading buffer, loaded into the wells and electrophoresed at 100 volts until the dye front approached the end of the gel.

5) The DNA was visualised on a UV transilluminator and photographed. The EtBr in the gel intercalates between the stacked base pairs of the DNA molecules in the gel and emits ultraviolet induce fluorescence.

2.1.2 Plasmid DNA

The expression constructs used for the transfections were pCI containing (i) a full length ret cDNA, a gift from Dr. V. Pachnis, (ii) ret/PTC2, an oncogenic form of Ret isolated from a papillary thyroid carcinoma (Bongarzone et al., 1993) a from Dr. I. Bongarzone. pCI contains a CMV promoter driving the expression of inserted DNA and an SV40 promoter driving expression of the neomycin resistance gene (neo\(^R\)), both in a sense orientation with respect to each other.

The pCI-ret/PTC2 construct was prepared by treating the pCDNA1-ret/PTC2 with Xba I to remove the ret/PTC2 insert. The pCI was also treated with Xba I and then calf intestinal phosphatase to prevent the plasmid from religating to itself. Both fragments were gel purified and cleaned as described below. The Ret/PTC2 fragment was then ligated into the Xba I sites of pCI. Orientation was confirmed by restriction mapping (data not shown).
The pCI-ret construct was prepared by treating pBS-ret with Sac II, Sal I and Pvu I to remove the full ret fragment and also cut the backbone in half to aid separation on the gel. The ret fragment was blunted with Klenow as described below and gel purified. The fragment was then ligated into Sma I site of pCI. Again orientation was confirmed by restriction mapping.

2.1.3 Sub cloning
1) 5 µg of backbone and 5 µg of insert were cut with the appropriate restriction enzyme for 1 hour at 37°C in 50 µl reaction.
2) The digest was run out on a 1 % LMP agarose gel.
3) The appropriate bands of linearised DNA were cut out of the gel and purified.
4) The purified DNA pellet was resuspended in 20 µl TE. 2 µl were loaded onto a 1 % agarose gel to quantify the DNA.
5) 100 ng of backbone were calculated and 500 ng molar equivalent of insert.
6) The DNAs were mixed alone with 1 µl T4 DNA ligase and 1 µl T4 DNA ligase buffer in 10 µl total reaction. This was incubated at room temperature for 2 hours. Backbone was also ligated alone as a positive control for the ligation reaction.
7) 5 µl of the ligation was used to transform competent E. coli which were spread onto LB agar plates containing 50 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ methicillin. The plates were then incubated over night at 37°C.
8) Colonies were grown up for small scale purification of DNA and screened for the insert.

2.1.4 Removal of 5' phosphate to prevent self ligation
Calf intestinal phosphatase (cip) removes the 3' phosphate to prevent religation of backbone. After the restriction digest in 50 µl, 5 µl of cip buffer and 1-2 µl of cip were added and incubated for 10 minutes at 37°C. These were then immediately run out on a 1 % agarose gel and purified.

2.1.5 Creating blunt ended DNA fragments from sticky ended fragments
After the restriction digest in 50 µl 1 µl of a 1 mM dNTP mix and 1 unit of the Klenow fragment of E. coli DNA polymerase (1 for each µg of DNA) was added. This was incubated for 15 minutes at room temperature. The enzyme was then inactivated by heating to 95°C for 10 minutes.
2.1.6 Recovery of DNA from an agarose gel

1) The DNA was run out on a LMP agarose gel containing ethidium bromide for visualisation under UV light.

2) Using a sterile blade the appropriate band of DNA was promptly excised from the gel, so as to minimize exposure of the DNA to the UV light. The gel was placed into a microfuge tube and the volume made up to 500 μl in ddH₂O. This was then heated at 65°C until the agarose had completely dissolved.

3) An equal volume of Tris saturated phenol was then added and vortexed to mix. It was then centrifuged for 2 minutes at 13,000 r.p.m. The upper aqueous phase was removed and placed into a new tube.

4) Step 3 was repeated twice.

5) An equal volume of chloroform was then added and vortexed to mix. It was then centrifuged for 2 minutes at 13,000 r.p.m. The upper aqueous phase was removed and placed into a new tube.

6) Step 5 was repeated once.

7) A 1/10 volume of 2M NaAc and 2.5 volumes EtOH were then added and vortexed and left at room temperature for 10 minutes. Finally they were centrifuged for 10 minutes at 13,000 r.p.m.

8) The pellet was washed in 70 % EtOH and left to air dry before resuspending in an appropriate volume of TE.

2.1.7 Making competent E. coli

1) 10 ml of LB were inoculated with a single colony of DH5α E. coli and were incubated overnight at 37°C with shaking.

2) 50 ml of LB were inoculated with 1 ml of the over night culture and incubated for 1 3/4 hours at 37°C with shaking. At this point the bacteria will be in the log phase of growth.

3) The culture was split into two sterile tubes and spun down at 2 K for 5 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 50 mM cold CaCl₂ gently. The bacteria were then left on ice for 40 minutes.

4) The bacteria were pelleted for 5 minutes at 2,000 r.p.m. The supernatant was discarded and the bacterial pellets were resuspended in a total of 4 ml of cold CaCl₂.

5) The bacteria could now be used in a transformation or they could be stored by adding 140 μl DMSO. After 15 minutes another 140 μl of DMSO was added and the cells aliquoted snap frozen and stored in liquid nitrogen.
2.1.8 Transformation of foreign DNA into bacterial cells

1) The transformation tubes were chilled on ice. For each transformation 50 µl of competent cells were used. 10 ng of plasmid DNA was then added and the tubes gently swirled to mix and left on ice for 45 minutes.

2) The cells were then heat-shocked for 45 seconds at 37°C and then snap chilled on ice.

3) 0.5 mls of LB were then added and incubated at 37°C for at least one hour with shaking to let the bacteria recover.

4) 50 µl of the culture were then plated onto LB agar plates containing the appropriate antibiotic. The plates were grown over night at 37°C in an inverted position.

2.1.9 Small scale preparation of plasmid DNA

Small quantities of DNA (5-20 µg) were obtained by the alkaline lysis method as described in Sambrook et al. (1993).

2.1.10 Large scale preparation of plasmid DNA

Large quantities (2-5 mg) of clean plasmid DNA were obtained by the alkaline lysis method and purified by ultracentrifugation through caesium chloride, as described in Sambrook et al. (1993).

2.1.11 Isolation of genomic DNA

DNA lysis buffer
10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)
100 mM NaCl
1 % SDS

Once made this buffer was stored at room temperature. Before use Proteinase K is added to a concentration of 0.1 mg ml⁻¹.

1) The medium was drained off a near confluent plate of cells. 2 mls of DNA lysis buffer was added and left in a 37°C incubator for about 5 minutes or until the cells have lysed and can be poured off the plate onto a 10 ml polypropylene tube.

2) The tube was incubated over night in a 55°C water bath to allow total digestion of all of the protein.

3) 2 ml of buffered phenol was added and the tube which was then mixed on a rotating wheel for 10 minutes before centrifuging at 3,500 r.p.m for 15 minutes.
4) The top aqueous phase was transferred into a fresh tube, being careful not to transfer any of the interface, before adding an equal volume of a 1:1 mix of phenol/chloroform. After mixing the sample was centrifuged as before.

5) Again the top layer was transferred into a fresh tube and an equal volume of chloroform was added. The sample was then mixed and centrifuged as before.

6) The top layer was again removed into a fresh tube and the DNA was precipitated in 2.5 volumes of EtOH and 0.1 volumes of 3 M NaAc. After gentle inversion of the tube the DNA becomes visible as a cotton wool-like precipitate. A sealed Pasteur pipette was used the pick out the DNA and put it in 200-400 µl of TE in an Eppendorf tube.

7) The DNA was left to dissolve at room temperature overnight and was then stored at 4°C.

8) Once dissolved the DNA was quantified using the spectrophotometer.

2.1.12 Southern blotting

1) The PCR products were loaded onto a 2 % agarose gel containing EtBr. The gel was run to separate the DNA according to size. The gel was photographed on a UV box with a ruler beside it for product size determinations.

2) Before blotting the DNA must be denatured in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 30 minutes at room temperature.

3) The gel was then neutralised in neutralising buffer (1 M Tris (pH 8.0), 1.5M NaCl) for 30 minutes, followed by a wash in 1 x SSC for 30 minutes.

4) During the last wash the apparatus for the transfer was prepared. An electrophoresis casting tray was placed upside down in a trough containing 10 x SSC. 2 pieces of Whatman 3MM paper were laid over the casting tray so that the ends were trailing into the buffer, thus acting as a wick. The gel was then placed on top of the wick. The membrane was cut to size and pre-wetted in 10 x SSC and placed on top of the gel. Three pieces of gel sized 3MM paper, pre-wetted in 10 x SSC were placed on top of the membrane and any air bubbles were removed by rolling the stack with a pipette. Saran Wrap is put around the edges of the gel to seal it, then a stack of tissue was piled on top and crowned by a gel plate holding a weight (usually a half full 500 ml bottle). This was left to transfer over night.

5) The following day the apparatus was dismantled and the position of the wells marked onto the membrane. The blot was washed for 5 minutes in 2 x SSC. Before storing the blot the DNA is fixed in a UV cross linker.
2.1.13 Preparation of $^{32}$P labelled probe

1) 25-50 ng of purified DNA fragment is boiled in a screw cap Eppendorf in a volume of 34 µl. 10 µl of oligo-labelling buffer, 5 U of Klenow polymerase and 5 µl of $^{32}$P dCTP were then added. The tube was incubated at 37°C for 1-2 hours.

2) A spin column was made for each individual probe as follows; 1 ml G25 Sephadex (buffered in TE) was pipetted into a 1 ml syringe plugged with glass wool and centrifuged inside a 10 ml polypropylene but at 1,700 r.p.m. for 4 minutes. 200 µl of TE was spun through the column under the same conditions. A screw cap Eppendorf was then placed under the column.

3) The probe volume was made up to 200 µl with TE. It was then added to the column with was centrifuged as before. The labelled probe was collected in the Eppendorf with the unincorporated $^{32}$P being trapped in the column.

4) To check that the labelling had worked the Eppendorf containing the probe was held next to a Geiger counter. The counts should be off scale when the tube was held 2-3 cm away. The probe was then used immediately.

2.1.14 Hybridisation of probe to Southern blot

1) The membrane was placed DNA side inwards in one of the oven's bottles. 20 ml pre-hybridisation solution (6 ml 20 x SSC, 1 ml 10 % SDS, 0.2 ml 0.5 M EDTA, 4 ml Denhardts, 10 % Dextran sulphate, 8.8 ml ddH$_2$O) was added and the bottle capped and put in the oven for at least 2 hours.

2) 3 mg of salmon sperm DNA was added to the tube containing the probe which was then boiled for 5 minutes.

3) The probe was then added directly to the pre-hybridisation solution and the blot was left to hybridise over night at 65°C.

4) The following morning the probe was poured away and the blot was washed in the bottle in 100 ml 2 x SSC, 1 % SDS for 30 minutes twice.

5) This was followed by a final wash in 0.1 x SSC, 1 % SDS for 30 minutes.

6) The membrane was the sealed in Saran wrap and put into a cassette with a piece of film and left over night at -70°C.

2.2 RNA protocols
2.2.1 Preparation of solutions, glassware and plasticware

Gloves were worn at all times and efforts were made to insure that the working area was dust-free. All solutions for RNA work were made, where possible, from solids which were kept separate from the general chemicals in the laboratory. Where handling was essential, chemicals were weighed out using baked spatulas. The solutions were then treated with 0.1% diethylpyrocarbonate (DEPC) at room temperature over night and autoclaved. DEPC is a potent inhibitor of RNase and is broken down to ethanol and CO$_2$ by autoclaving. The only aqueous solutions which were not DEPC-treated were those containing Tris or amines and those which could not be autoclaved. These solutions were made up in bottles which had been DEPC treated and baked.

All glassware, spatulas, and homogenisers were baked over night at 180°C before use. Sterile plasticware was assumed to be RNase-free.

2.2.2 Extraction of total RNA from mouse and human tissues

Total RNA was extracted from mouse and human tissues using TRI REAGENT™ (Molecular Research Centre, Inc), which is based on the guanadinium thiocyanate/phenol methodology of RNA extraction of Chomczynski and Sacchi, (1987). Using this procedure it was possible to extract total RNA from tissue in under 2 hours. TRI REAGENT™ promotes formation of RNA complexes with guanadinium and water molecules and inhibits hydrophilic interactions of DNA and proteins. As a consequence, DNA and proteins are excluded from the aqueous phase, leaving the RNA which can then be purified. The RNA is of high quality and contains the whole range of cellular RNA molecules.

Mouse embryos were collected at various stages of gestation and placed in ice-cold DEPC treated PBS, they were then dissected within and hour of collection.

RNA was extracted from the dissected organs as follows:

1) The tissue was homogenised in TRI REAGENT™ by passing it through a 23 gauge needle several times. 1 ml of TRI Reagent was used per 50-100 mg tissue. For extraction of the RNA from the ureteric bud and mesenchyme dissections 500 μl of TRI Reagent was used along with 1 μl of glycogen which acted as a carrier, making the RNA easier to precipitate and visualise.

2) 200 μl of chloroform was then added and the tubes mixed vigorously. The samples were then stored at RT for 10 minutes.

3) The tubes were then spun for 15 minutes at 13,000 r.p.m. at 4°C.
4) The aqueous top phase was transferred into a new tube and 0.5 ml of propan-2-ol was added. The tubes were vortexed, stored for 10 minutes at room temperature and then centrifuged for 15 minutes at 13,000 r.p.m. at 4°C.

5) The supernatant was discarded and the pellet washed in 70% ethanol.

6) The pellet was air dried and resuspended in DEPC treated water and stored at -70°C.

2.2.3 Extraction of total RNA from cells grown in monolayer

Cell monolayers were washed twice with ice cold PBS. The cells were lysed directly in the culture dish upon the addition of 1 ml TRI REAGENT per 10 cm dish. The cells were scraped into an Eppendorf using a plastic policeman and homogenised by passing the lysate through a 23 gauge needle several times. The remainder of the protocol was as described above for tissues.

2.2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.2.4.1 Introduction

Reverse transcription involves the production of complementary double stranded DNA using single stranded RNA as a template and a random eight base primer. This process is done using the enzyme reverse transcriptase (RT) isolated from a retrovirus. This cDNA is then suitable for amplification by the polymerase chain reaction (PCR).

PCR allows the selective amplification of target DNA sequences. The process requires oligonucleotide primers, one for each DNA strand, on either side of the target region. PCR relies on cycles of denaturation of the double stranded DNA to give single strands, primer annealing to their complementary sequences and synthesis of new complementary DNA strands. Successive repetition of this process results in the specific amplification of the region bounded by the primers.

These two techniques when combined together give a very powerful detection system that can be applied to very small samples, such as embryonic organs, or used when expression levels are very low. The amplification of β-actin is used as a control for the integrity of the RNA sample used in each RT reaction.

2.2.4.2 Reverse transcription

1) 1.5 μg RNA in a volume of 8.5 μl of DEPC water was heated to 95°C for 5 minutes and then snap chilled on ice.

2) A pre-mix was made for each sample containing: 1st strand buffer 4 μl, dNTP (2.5 mM) 2 μl, RNA guard 0.5 μl, RT 1 μl, 0.1 M DTT 2 μl, DEPC H₂O 1 μl, and 1:15 hexamers 1 μl.
3) 11.5 μl was added to each sample. The tubes were incubated at room temperature for 5 minutes then at 37°C for 1 hour.

4) 30 μl DEPC treated water was added to each tube which was then heated to 95°C for 10 minutes and snap chilled on ice. 2.5 μl of this reaction was then used in the following PCR reaction, the remaining sample could be stored at -20°C.

### 2.2.4.3 PCR Reaction

PCR reactions are reliant on the specific binding of the primers to the homologous regions of RNA. Within the PCR reaction several factors influence this binding specificity and for this reason the annealing temperature and magnesium chloride concentrations needed for each set of primers was optimised to give amplification of a single RNA species. The results are described in Chapter 3.

1) A pre-mix was made for each sample containing: 10 X NH₄ buffer 5 μl, MgCl₂ 1 or 1.5 μl, dNTP (2.5 mM) 1 μl, 5' primer 1 μl, 3' primer 1 μl, Taq 0.5 μl, and ddH₂O 37.5 μl.

2) 47.5 μl was added to each tube giving a total volume of 50 μl.

3) The amplification times were as follows: a primary denaturation step of 94°C for 5 minutes followed by 30 cycles of: a denaturation step, 94°C for 1 minute, an annealing step, 65°C for 1 minute for GDNF and 60°C for 1 minute for all the other primers and a primer extension step, 72°C 1 minute, with a final extension of 72°C for 10 minutes.

4) 10 μl of the PCR product was electrophoresed through 2% agarose gel.

5) The identity of the PCR products was confirmed by size, restriction mapping and Southern blotting followed by hybridisation with a confirmed cDNA probe.

### 2.2.5 Whole mount in situ hybridisation

In situ hybridisation is a technique by which labelled probes are hybridised to tissue thereby revealing the cellular localisation of the related mRNA within the tissue. The negative control is in the form of non-specific probes. This methodology is based on Wilkinson et al., (1987). The probes used were pmcrest 7 and GDNF1.8 (kind gifts from V. Pachnis, NIMR) for Ret and GDNF. For GDNFRα a Wash-U Merck I.M.A.G.E. clone was used, with sequence identity to GDNFRα, clone ID 402011.

In situ hybridisation was undertaken as described in Wilkinson (1992). When sufficient coloured reaction product had developed (a few hours to overnight), embryos were washed in several changes of PBS.
2.2.6 Vibrotome sectioning

1) Embryos larger than E10 need to be pre equilibrated in the embedding mix over night.
2) Fill mould with embedding mixture. Add 1/10 by volume of 25 % glutaraldehyde solution and mix quickly. Place embryo into mould and orientate quickly. The mix will set in less than 1 min. Leave for 1 hour to set fully.
3) Trim block and mount onto vibrotome stage. Bath in PBT and cut sections 50-100 μm thick. Place on slide and mount in 80 % glycerol PBT.
4) Visualise and photograph using Zeiss Axiophot under Differential Interference Microscopy.

2.3 Protein protocols

2.3.1 Protein extraction for Western blotting

**RIPA Buffer**

- 150 mM NaCl
- 1 % NP40
- 0.5 % DOC
- 0.1 % SDS
- 50 mM Tris (pH 8)

Before use the following protease inhibitors were added:

- 1 mM PMSF
- 30 μg ml⁻¹ Aprotinin
- 1 mM sodium orthovanadate

1) Adherent cells must not be trypsinised but scraped off using a plastic policeman. The minimum amount of RIPA (100 μl for a confluent 10 cm plate) was added and this would lyse the cells. Once lysed the samples were kept on ice for 15 minutes.

2) The lysates were then centrifuged at 14,000 r.p.m. at 4°C for 15 minutes. The pellet was discarded and the lysate was then ready for western blotting or immunoprecipitation.

2.3.2 Immunoprecipitation

In this procedure, antibody is incubated with a cell lysate, and specific antibody-antigen complexes are collected by precipitation with Protein A-agarose. Precipitated proteins can then be analysed by SDS-polyacrylamide gel electrophoresis.
1) 500 µl of crude protein extract was precleared with 2 µl of a 1:20 normal rabbit IgG for 1 hour at 4°C. Preclearing helps to minimise the background caused by non-specific binding of extraneous cellular proteins to the Protein A-agarose.

2) The protein A-agarose beads were washed in 1 ml RIPA buffer and then pelleted twice before being made back up to their original volume. 20 µl of the washed bead were added to the lysates and incubated for 30 minutes. The beads were then pelleted for 15 minutes at 4°C.

3) The supernatant was placed into a fresh tube and 50 µl Groves, 2 µl Bill 2 was added. The antibody complexes were left to form at 4°C for 1 hour. 20 µl of washed agarose beads were then added and incubated for 30 minutes at 4°C.

4) The protein precipitates were collected by centrifuging the samples for 5 minutes at 4°C.

5) The supernatant was discarded and the pellet washed three times in 1 ml RIPA.

6) To the washed pellet 50 µl electrophoresis loading buffer was added and boiled for 5 minutes. Before loading onto the SDS-PAGE gel the samples were centrifuged for 5 minutes to pellet the beads.

2.3.3 Western blotting

Western blotting is the technique of transferring proteins to nitrocellulose filters. The proteins can then be visualised using a coomassie blue stain or probed using a specific antibody. The proteins can be denatured or kept in their native form before being separated on an SDS-polyacrylamide gel. The proteins are thus separated according to size with the smaller proteins migrating the farthest.

2 x Loading buffer

1 ml glycerol
0.5 ml β-mercaptoethanol
3 ml 10 % SDS
1.25 ml 1 M Tris (pH 6.7)
1-2 mg bromophenol blue

Once made this was stored in frozen aliquots.

1) The gel apparatus used was the BioRad Mini-Protean II and was set up according to the manufacturers instructions. 6 % gels were used to separate the 150 and 170 kDa ret proteins. The running and stacking gels were made according to the recipes (page). The running gel is made first, with the ammonium persulfate and TEMED added last as they cause the polymerisation of the acrylamide by providing the free radicals. Whilst setting the running gel
is overlaid with water saturated butan-1-ol to prevent oxygen diffusing into the gel and inhibiting polymerisation.

2) Once set the butan-1-ol was washed off the stacking gel made and poured with a clean comb inserted into the top. The stacking gel is of high porosity and acts to stack the proteins in a very thin layer on the interface between the two gels. Once set the gel running apparatus was assembled and filled with running buffer.

3) An equal volume of loading buffer was added to each protein sample and boiled for 10 minutes. The samples were put straight on ice to cool and then spun for 1 minute to remove any large debris. They could then be loaded onto the stacking gel.

4) The proteins were run through the stacking gel at 50 V until the dye front had just moved into the running gel, the voltage was then turned up to 160 V. The gel was run until the dye front had just run off the running gel into the running buffer.

5) The apparatus was disassemble and the glass plates, holding the gel, prised apart. The stacking gel was removed and discarded, the running gel was placed into transfer buffer (5.8 g Tris, 2.9 g glycine, 3.75 ml 10 % SDS, 200 ml methanol) for 10 minutes.

6) One piece of nitrocellulose and 6 pieces of Whatman 3MM paper were cut to the exact size of the gel and pre-wetted in transfer buffer.

7) The proteins were transferred from the gel to the nitrocellulose filter using a semi-dry electroblotter set up in the following way; 3 pieces of the 3MM paper were placed on the anode, the gel was carefully floated onto the nitrocellulose membrane was then placed on top of the 3MM paper, with the gel uppermost. Finally the remaining 3 pieces of 3MM paper were placed on top of the gel. The whole assembly was rolled out with a pipette to remove any air bubbles. The lid which contained the cathode was then fitted and 12 V was passed through the system for 1 hour.

8) The transfer apparatus was then carefully dismantled and the top left hand corner of the membrane marked for orientation purposes. The gel was discarded and the filter placed in a solution of Ponceau S. This is a dye that stains proteins and shows the proteins have indeed passed from the gel onto the membrane, it does not, however, interfere with the immunological detection methods which follow. The Ponceaus S was washed off with ddH$_2$O before proceeding onto the immunological detection.

2.3.4 Immunological detection

1) Membranes were blocked in TBS containing 0.1% Tween 20 and 5% skimmed milk for 1 hour at room temperature. This blocks the non-specific binding sites.

2) The membranes were then incubated in the primary antibody, which had been diluted in block solution (B2 1:500, A1 1:5), and were incubated at 4°C over night.
3) The primary antibody was washed off for three 15 minute periods in TBS.

4) The secondary antibodies were conjugated to horseradish peroxidase and were used at a concentration of 1:2000, in blocking solution. This was left on the membranes for 1 hour at room temperature.

5) The secondary antibody was washed off with TBST in three 15 minute washes.

6) To detect the protein the membranes were exposed to Enhanced Chemi-Luminescence reagent for 1 minute. Excess reagent was drained and the membranes was wrapped in Saran Wrap and exposed to X-ray film for 30 seconds to 30 minutes, depending on the intensity of the signal. Proteins were sized by comparison with Rainbow markers.

2.3.5 Staining the blot with India ink

This method of staining is very sensitive and yields a permanent record. It relies upon the preferential adherence of the colloidal carbon particles in India ink to the immobilised protein on the filter.

1) The blot was washed in 0.4 % Tween 20 in PBS, with two changes at 5 minutes each.

2) The ink solution was made up (100 μl ink in 100 ml of 0.3% Tween 20 in PBS) and the blot placed into it.

3) The blot was incubated at room temperature for 15 minutes to 18 hours.

4) Once stained the blot was washed in multiple changes of PBS.

2.3.6 Staining the gel with Coomassie Brilliant Blue R-250

1) The gel was fixed for 2 hours in; 50 % MeOH, 10 % acetic acid and 40 % ddH₂O.

2) Once fixed the gel was stained in 50 % MeOH, 0.05 % Coomassie Brilliant Blue R-250, 10 % acetic acid and 40 % ddH₂O, for 4 hours.

3) After staining the gel was destained in; 5 % MeOH, 7 % acetic acid and 88 % ddH₂O until the background was clear.

2.4 Immunohistochemistry

This technique involves the identification of molecules in situ by means of a specific antigen-antibody reaction associated together with a means of visualisation.

Two types of antibody have been used in this study; monoclonal antibodies and polyclonal antibodies. Antibodies are produced as part of the immune response to a foreign antigen. Polyclonal antibodies are easier to produce, usually in rabbit, but also commonly in
pigs, sheep, and goats. The procedure involves injecting an adult animal with the antigen of interest, over a period of several months. Antigen-specific B-lymphocytes are stimulated when antigen binds to cell surface receptors, resulting in the proliferation of the antigen-stimulated B-lymphocytes and the secretion of large amounts of antibody.

Each B-lymphocyte gives rise to a clone of daughter cells. However, many different B-lymphocytes will be stimulated by a variety of antigenic determinants giving a mixed population of secreted antibodies. This is said to be polyclonal. Monoclonal antibodies are produced by fusing activated B-lymphocytes with myeloma B-lymphocytes and cloning the hybrid cells. Once immortalised these cells will produce and unlimited amount of monoclonal antibody. Mice and rats are used for this technique.

2.4.1 Immunocytochemistry of tissue culture cells in monolayer
1) Cells were seeded into 8 well chamber slides and grown until near confluency.
2) The growth medium was removed and the chambers washed twice in PBS followed by 4 % PFA or ice cold methanol to fix for 5 minutes.
3) After a blocking step of 10 % goat serum for 1 hour at room temperature the cells were exposed to the primary antibody diluted appropriately in block overnight at 4°C. Negative controls were left in block overnight and then treated the same as the experimental slides.
4) After washing off the primary antibody with several changes of PBS an FITC antibody was applied for several hours at room temperature.
5) Finally the secondary antibody was washed off and the slides mounted in PBS before analysis and photography on a Zeiss Axiophot microscope.

2.4.2 Whole mount immunohistochemistry
1) The rudiments were fixed in 4 % PFA at room temperature for 30 minutes. The tissues were permeabilised by adding 0.01 % Triton X in the wash steps but not in the fix.
2) The rudiments were washed with PBS for 10 minutes three times.
3) 10 % FCS in 1 % BSA was used to block the rudiments for 40 minutes.
4) The block was then removed but the organs were not washed at this stage.
5) The primary antibody was diluted to an appropriate concentration in block and incubated over night at 4°C.
6) The primary antibody was washed off in PBS in three 10 minutes washes.
7) The secondary antibody in diluted in block and filtered through a 0.22 μm filter to remove any particles. The rudiments were incubated in the secondary antibody over night at 4°C.
8) The antibody was washed off in at least 3 x 10 minute washes in PBS, longer washing, up to over night gave much cleaner results.
9) The rudiments were mounted in Citifluor™ using a pipette with a cut off tip. When the samples were to be looked at using the confocal microscope the coverslips had to be sealed down with nail varnish.

2.4.3 Counterstaining with propidium iodide

Propidium iodide stains the nucleic acids within the cell. The addition of RNase ensures that only the DNA, and hence the nucleus stains, allowing the visualisation of the tissue structure.

1) After washing off the secondary antibody the rudiments were incubated in 2-4 µg ml⁻¹ PI plus 100 µg ml⁻¹ RNase (DNase free) at 37°C for 30 minutes in the dark.
2) The excess PI was washed off in PBS in 3 x 10 minute washes and mounted as described above.

2.5 Histological analysis

2.5.1 Fixation, embedding, and sectioning of tissues

1) Tissues were fixed in 4 % PFA made up in PBS and left for 1 hour to overnight depending on the size of the tissue.
2) The tissues were then dehydrated through a series of ethanols for 30 minutes each, at room temperature: 30 %, 50 %, 70 %, 85 %, 95 %, and 100 % twice.
3) They were then placed into two changes of Histoclear for 30 minutes each.
4) The Histoclear was replaced with a 1:1 mix of Histoclear:wax at 60°C for 1 hour.
5) After three changes of wax, each at 60°C for 30 minutes the samples were transferred to a mould, where they were orientated and the left to set.
6) 6 µm sections were cut and placed on to clean glass slides, and stored at room temperature until use.

Frozen sections were cut using a cryostat. Tissues could be cut fixed or unfixed. The tissues were embedded in oct on a cryostat chuck and stored at -20°C until the embedding medium had frozen. The chuck was then positioned in the cryostat and sections of 6 µm were cut at -20°C. The sections were collected onto gelatine-coated slides, air dried, and stored at 4°C.
2.5.2 Hematoxylin and eosin (H and E) staining

1) Sections were dewaxed in Histoclear for 15 minutes, followed by immersion in 100 % EtOH twice for 2 minutes.
2) The slides were rehydrated through an ethanol series of 95 %, 85 %, 70 %, 50 %, and 30 %, for 1 minute each before a final wash in running tap water.
3) The slides were dipped in 0.1 % Harris hematoxylin for 2 minutes, followed by a rinsing in running tap water for 5 minutes: this blues the sections.
4) The slides were then dipped in 1 % aqueous eosin for 1 minute, followed by a rinsing in running tap water for 5 minutes: this pinkens the sections.
5) The slides were then passed back through the ethanol series into 3 changes of Histoclear, to remove any remaining ethanol and allowed to air dry before mounting.

2.6 Tissue culture

Transgenic mouse cell lines were grown in Dulbeco's Modified Eagle Medium supplemented with interferon and 5 % fetal calf serum. All other cell lines were grown in supplemented with 10 % fetal calf serum, Penicillin-streptomycin (P/S, 1000 U ml⁻¹, Gibco)

2.6.1 Transfection of cells in monolayer

In this procedure an expression vector containing a gene is introduced into tissue culture cells. In this case I have used the Lipofectin reagent. Lipofectin reagent interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells results in the efficient uptake and expression of the DNA.

1) 1-2 x 10⁵ cells were seeded on to a six well tissue culture plate in the appropriate growth medium supplemented with serum.
2) The cells were incubated under normal conditions over night.
3) Solution A and B were prepared as follows;

Solution A

For each transfection 1 μg of DNA was diluted into 100 μl of Opti-mem™.

Solution B

For each transfection 10 μl of Lipofectin reagent was diluted into 100 μl Opti-mem™.
4) Solution A and solution B were combined, mixed gently and incubated at room temperature for 10 minutes.
5) During the above incubation the cells were washed once with 2 ml Opti-mem™.
6) For each transfection 0.8 ml Opti-mem™ was added the A-B solution. After mixing gently 1 ml was overlaid onto each well of the six well tissue culture plate.
7) The cells were incubated over night under normal growth conditions.
8) The DNA containing media was replace with normal growth media containing serum and the cells were grown for 48 hours.

2.6.1.1 Optimisation for seeding cell number

Transfections with Lipofectin are very dependent on cell confluency at the time of transfection. For this reason the cell number was optimised using a construct with a lac Z gene as a reporter gene, pSDKlacZpA. Cells were seeded from $7.5 \times 10^4$ to $4 \times 10^5$. After 24 hours the cells were transfected and then left for two days before staining with X-gal. It was found that the optimal concentration for the M5 and P1 cells was $3 \times 10^5$ and for NIH3T3 and MDCK cells $1 \times 10^5$.

2.6.2 Selection of positive transformants

The expression vector used contained neomycin resistance so positive transformants were selected for by the addition of G418 to the growth media. To establish the concentration of G418 needed to kill all untransformed cells it was necessary to titrate the G418 with the cells to be transformed.

2.6.2.1 Optimisation of G418 concentration

Cells were seeded subconfluently and left over night to attach. The following day a range of G418 concentrations from 0.25 mg ml$^{-1}$ to 1 mg ml$^{-1}$ were diluted in normal growth medium. The cells were then grown in this medium for 2 weeks, the medium was replaced every 2-3 days. By 2 weeks the minimum concentration that would kill all of the cells was assessed and this concentration was used to select transformed cells which were subsequently seen as small isolated colonies.

2.6.3 [³H]Thymidine incorporation by cells

[³H]Thymidine incorporation was determined after cloning under normal growth conditions and with the addition of GDNF. It was used as a marker of cellular proliferation by labelling the dividing cells with [³H]Thymidine.
1) Cells were plated in 96 well plates at an initial density of 4,000 cells per well. If GDNF was to be added it was added once the cells had attached to the well. The cells were then left to grow for 48 hours.

2) $^{3}\text{H}$Thymidine was added to the medium at 1 mCi ml$^{-1}$ and the cells were grown for another 24 hours prior to collection of filter discs using an automatic cell harvester. The discs were washed, dried and analysed by liquid scintillation counting.

2.6.3.1 Optimisations of seeding cell concentration and day of $^{3}\text{H}$thymidine pulse

This protocol relies on the cells being in log phase when they are pulsed with $^{3}\text{H}$thymidine. If the cells are too few and therefore not proliferating at their maximal rate, or too many, and therefore undergoing contact inhibition, the growth profile seen will be inaccurate. For these reasons both the seeding cell number and the day of pulsing with $^{3}\text{H}$thymidine was optimised. Results are shown in chapter 5.

2.6.4 Three dimensional cultures in collagen gel and Matrigel

Collagen gels and Matrigel are used to grow cells in a three dimensional culture system (Montesano et al., 1991). Under these conditions the cells can show a branching phenotype which is not seen under normal two dimensional culture systems.

Cells were resuspended in the appropriate gel at a concentration of 10,000 cells ml$^{-1}$. The gel was then left to set at 37°C for 15 minutes. A layer of growth medium was then laid over the gel. The gels could be kept going for several weeks with the medium being changed every 2-3 days.

2.6.5 Scatter bioassay

This test is used to confirm the bioactivity of HGF/SF on MDCK cells, a cell line highly sensitive to scattering. This assay has been developed based on the findings of Stoker and Perryman, (1985), Stoker et al., (1987), and Gherardi et al., (1989). MDCK cells are an epithelial cell line which normally grow in compact, adherent epithelial cell islands, but disperse or 'scatter' upon the addition of HGF/SF. D4-ras NIH3T3 secrete high levels of HGF/SF and were used as a positive control to 'condition' the media.
1) D4-ras NIH3T3 cells were grown to confluency. The growth medium was replaced and left for 2 days to be 'conditioned'.

2) Serial doubling dilutions of the conditioned medium (0.15 ml) were added to a suspension of 3 x 10^3 MDCK cells in 0.15 ml growth medium and were seeded into a 96 well plate and grown overnight.

3) The following day the cells were washed twice with PBS and fixed in 4 % PFA in PBS for 10 minutes and washed again.

4) To visualise the cells they were stained with Coomassie Brilliant Blue R-250 (0.05 % Coomassie Brilliant Blue R250, 20 % MeOH, and 7.5 % glacial acetic acid) for 20 minutes before being washed off with PBS.

5) The phenotype of cells was then recorded.

2.6.6 Aggregation assays

It was found that if cells were prevented from attaching to the plastic by a thin layer of agar they would aggregate into spherical aggregates. This is an interesting in vitro model for looking at cell differentiation.

1) 10 cm tissue culture dishes were coated with a thin layer of agar made up in tissue culture grade H_2O, left to set and then equilibrated with growth medium.

2) A near confluent plate of cells was harvested and split onto four coated plates. The cells were left to aggregate from over night to a week.

3) Aggregates were harvested by letting them sink to the bottom of a falcon tube before removing the medium.

2.6.7 Cell migration assay

Cells were grown to confluency in a 6 well plate. A series of artificial wounds were made through cell monolayers using a plastic pipette tip. Cells were then incubated whether in the absence or presence of 100 ng ml^{-1} rhGDNF for 24 hours. Cells were finally fixed with 4 % PFA and stained with Coomassie Blue solution, as described for scatter assays. Cells were then observed with an inverted microscope.

2.6.8 X-gal assay

1) The medium was removed from the plate of cells and was then washed with PBS once.

2) The cells were fixed with 0.5 % glutaraldehyde in PBS for 15-20 minutes.

3) The fix was washed off with two washes in PBS.
4) The X-gal solution was filtered and poured onto the plate. The plate was then incubated for 2-4 hours at 37°C, when a blue colour could be seen in some of the cells.

**X-gal buffer**

100 mls PBS  
0.01 % Na Deoxycholate  
20 µl NP40  
0.04 g MgCl₂ (2 mM)  
0.165 g Potassium fericyanide (5 mM)  
0.211 g Potassium ferrocyanide (5 mM)  
Store at 4°C and filter before use.

**Stock X-gal**

50 mg ml⁻¹ X-Gal in dimethylformamide. Store in glass in the dark at -20°C.

**X-gal solution**

Add X-gal stock to X-gal buffer to give a final concentration of 1 mg ml⁻¹ (0.12 mls in 6 mls per plate). The solution was filtered just prior to use.

### 2.7 Organ culture

Organ culture is an in vitro system which allows an organ to grow and differentiate in culture. Grobstein used organ culture systems to look at the development of many branching organs (Grobstein, 1953). In this present study kidneys, lungs, and submandibular salivary glands have been grown in culture.

Rudiments were randomly assigned to control or treatment groups and transferred to Biopore tissue culture inserts in 6-well plates. Rudiments were cultured in DMEM/F12 supplemented with 5 % FBS, and penicillin-streptomycin at 37°C, under high humidity in an atmosphere of 95 % air/5 % CO₂. The nutrient medium was changed completely every 48 hours. Recombinant human GDNF was reconstituted as recommended by the manufacturer at 100 mg ml⁻¹ in sterile tissue culture grade water and stored frozen in small aliquots. This stock solution was further diluted to give a working stock of 10 µg ml⁻¹ and added to the medium just before use, control cultures had nothing added. Specific neutralising antibodies against recombinant human GDNF (goat anti-rhGDNF) or control normal goat IgGs were
reconstituted in sterile PBS at 1 mg ml\(^{-1}\) and stored in small aliquots at -20°C. Two or three separate experiments were performed for each organ and treatment, with 5-20 rudiments in each treatment group. The morphology was documented with photomicrographs using an inverted microscope.

The lung cultures were grown for 4 days, the submandibular salivary glands for 3 days and the kidney for 2 days. After this time the organs were fixed for 30 minutes in 4 % PFA in PBS before being stained with anti laminin antibodies. Subsequent analysis used the confocal microscope.

2.8 Materials

2.8.1 General reagents

All reagents apart from those listed below were Analar grade supplied by BDH Ltd. Those listed below were supplied by Sigma Chemicals Co: Trizma base, BSA, ethidium bromide, Coomassie blue, sephadex G-50, 2-mercaptoethanol, N-lauroylsarcosine, bromophenol blue, orthovanadate, phenylmethylsulphonyl fluoride, aprotinin, ampicillin, methicillin, paraformaldehyde, phenol, X-gal, IPTG, DEPC, TEMED, Ponceau S, concentrated glutaraldehyde, and proteinase K.

dATP, dCTP, dGTP, dTTP (Promega Corp.). TRI REAGENT™ (Molecular Research Centre, Inc.). Histoclear (National Diagnostics). Lennox broth and Lennox broth agar (Difco Laboratories). PBS (Gibco BRL). Protein A-agarose (Santa Cruz Biotechnology). Dig-RNA labelling mix, Nibroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl (Promega).

2.8.2 Tissue culture equipment and media

All cell and organ culture media and supplements were supplied Gibco BRL. All tissue culture plastics were supplied by Philip Harris and Nunc Inc. Millicell filters (Millipore Corp.). Recombinant murine \(\gamma\)-interferon, insulin-like growth factor (Genentech). Dulbecco’s modified eagle medium, without sodium pyruvate, with 4500 mg/L glucose and Dulbecco’s MEM nutrient mix F-12 (HAM) with L-glutamine, with 15 mM HEPES (Gibco).

2.8.3 Sources of mouse tissues and cells

Outbred CD1 mice (Charles River Mouse Farms) were mated overnight and the morning after designated as day 0.5 of gestation. Pregnant mice were killed by cervical dislocation on embryonic day (E) 10.5 through to 16.5, and tissues were removed under aseptic
conditions and place in L15 medium (Gibco). Organ rudiments were isolated from embryos by microdissections at E11.5 (metanephros), late E10 (lung), or late E12 (salivary gland). Metanephroi were usually dissected free of the Wolffian duct however in one experiment the duct was left intact to examine its affect on the treatments in that study (chapter 4). Tissues for RT-PCR analysis and in situ hybridisation were isolate by microdissection and placed into DEPC treated PBS.

Mardin Darby canine kidney (MDCK) cells and D4-ras 3T3 cells were obtained from Dr. Ermano Gherardi, ICRF, Cambridge, NIH 3T3 cells from Dr. Jonathan Stoy, NIMR, London. M5, P1, MC2, MC3, MC4, MC5, MC9, MC10, MC11, MC12, MC13 and A1-2 cells were obtained from Dr. Adrian Woolf, ICH, London.

M5 cells were derived from the metanephric mesenchyme of an E11 mouse. They are vimentin positive and cytokeratin , negative and are indicative of an early uninduced mesenchyme cell (Woolf 1995)

2.8.4 Enzymes


2.8.5 Photography, autoradiography, and blotting


2.8.6 Radioisotopes

[methy-] Thymidine 5 Ci/mmol (Amersham). [α] dCTP 3000 Ci/mmol (ICN).

2.8.7 Gel electrophoresis

Agarose and low melting point agarose (Gibco BRL). Protogel (37.5:1 polyacrylamide to bisacrylamide stabilised solution).

2.8.8 Bacterial strains

DH5α (E. coli) (Promega).
2.8.9 Molecular size markers and cloning vectors


2.8.10 Oligonucleotides

GDNFRα sense primer, 5' ATTGGCACAGTCATGACTCCCAAC 3' corresponding to nucleotides 1177-1201 of mouse GDNFRα cDNA.
GDNFRα antisense primer, 5' GAGGAGCAGCCATTGATTTGTGG 3' corresponding to nucleotides 1598-1622 of mouse GDNFRα cDNA. Accession number U59486 (Jing et al., 1996).

GDNF sense primer, 5' CGCTGACCAGTGACTCCAATATGC 3' nucleotides 253-277.
GDNF antisense primer 5' ACATTGTCTCGGCCGATTCACAGG 3' nucleotides 442-466. Accession number U37372 (Hellmich et al., 1996)

Ret sense primer, 5' GGCCTCTATTTCTCAAGGGATGCT 3' corresponding to nucleotides 82-106 of mouse Ret cDNA.
Ret antisense primer, 5' GATGTTGGGACAAAGGAACTGCAC 3' corresponding to nucleotides 576-600 of mouse Ret cDNA. Accession number X67812 (Iwamoto et al., 1993).

ret/PTC2 sense primer, 5' TTCGAGAATGTGAGCTCTACGTCC 3' corresponding to nucleotides 53-77 of human ret/PTC2.
ret/PTC2 antisense primer, 5' CTTCATCACCTTGCTGAATCACAG 3' corresponding to nucleotides 425-449 of human re/PTC2. Accession number L03357 (Bongarzone et al., 1993).

β-actin sense primer, 5' GTGGGCCGCTCTAGGCACCAA 3' corresponding to nucleotides 24-45 of mouse β-actin cDNA (Clontech).
β-actin antisense primer, 5' CTCTTTGATGTCACGCACGATTTC 3' corresponding to nucleotides 540-564 of mouse β-actin cDNA (Clontech).

Synthesis occurred from the 3' end to the 5' end. The most 3' base is attached to a solid support and successive bases added. The GDNFRα, GDNF and Ret oligonucleotides were synthesised by Genosys UK. The β-actin primers were obtained form Clontech.
2.8.11 Plasmid DNA

Mouse Ret cDNA and mouse GDNF cDNA were obtained from Dr. Vassilis Pachnis, NIMR, London. Mouse GDNFRα was obtained from the HGMP Resource Centre, UK, EST 402011. pSDKlacZpA was a kind gift from Dr. Debora Henderson, ICH, London. pCI-neo was a kind gift from Dr. Jonathon Stoy, NIMR, London.

2.8.12 Western blotting equipment

Mini-Protein apparatus, Trans-blot SD semi-dry transfer cell (BIO-RAD).

2.8.13 Antibodies

Antibody SCI was raised in rabbits against a synthetic peptide consisting of the carboxy-terminal 20 amino acids of ret and was a gift from Dr. Masahide Takahashi, Noyaya University.

Antibodies B1 and B2 were a gift from Dr. Marc Billaud. These antibodies were raised in rabbits against the carboxy-terminal 20 amino acids of ret.

Antibody A1 was a cocktail of three monoclonal antibodies raised in Chinese hamsters against the 20 5' amino acids of ret and was a gift from Dr. Andrew Groves, Caltech, Pasadena.

2.8.14 Centrifuges

In all cases samples were centrifuged in Eppendorf tubes in a Heraeus Biofuge 13R, unless otherwise stated.

Anti-human GDNF neutralizing antibody was raised in goats to purified, E.coli-derived, recombinant human GDNF (R and D systems AB-212-NA).

Anti-laminin antibody was raised in rabbits to repeated injections of purified laminin (Sigma L9393)
3. Expression of ret, GDNFRα, and GDNF during mouse embryogenesis

3.1 Introduction

Recent studies have shown that GDNF-induced activation of ret is mediated by GDNFRα (Jing et al., 1996; Treanor et al., 1996). Ret and GDNF are expressed from E8.5 of mouse development in patterns consistent with multiple roles in neural differentiation and epithelial-mesenchymal interactions (Pachnis et al., 1993; Hellmich et al., 1996). Activation, and subsequent phosphorylation, of ret is dependent upon the coexpression of GDNFRα. Expression of GDNFRα may, therefore, add a further level of cellular control over the activation of ret by GDNF. For this reason, the expression patterns of the individual components of the ret-GDNF signaling system may show subtle differences, both spatially and/or temporally.

A review of the expression data published prior to the identification of GDNF as the ligand for ret did not reveal a cohesive story. For this reason a comparative investigation into the expression patterns of ret, GDNF, and GDNFRα during development, was undertaken. RT-PCR was used as an initial, and highly sensitive screening method to look at expression of the genes in organs that develop by branching morphogenesis. These results were confirmed using dig-labeled riboprobes on whole mount in situ hybridisations. RT-PCR has the advantage of being very sensitive and is capable of detecting gene expression in the tiny amounts of RNA extracted from embryonic organs. Its sensitivity, however, is also a potential pitfall as it is possible to get false positives though contaminating tissue. However, positive results were confirmed using in situ hybridisation and all experiments were controlled for both RNA and DNA contamination. In addition, in situ hybridisation allowed the localization of specific cell populations which were expressing the genes of interest.

To correlate protein expression patterns with the mRNA expression seen by in situ hybridisation, whole mount immunohistochemistry was performed on micro-dissected metanephros, lung, and salivary glands using antibodies raised against epitopes of the ret protein. These organs were then visualized using the confocal microscope. Four ret antibody preparations were used in this study, but unfortunately, none gave a specific binding pattern on either Western blots or whole mount organ preparations.
Finally, in order to investigate further the expression of ret, GDNFRα, and GDNF in the renal mesenchyme, cell lines derived from the early metanephric mesenchyme (Woolf et al., 1995) were screened for expression of ret, GDNFRα, and GDNF by RT-PCR.

3.2 Results

3.2.1 RT-PCR optimization

The annealing temperature and concentration of MgCl₂ dramatically alter the specificity of the primer binding dynamics in a PCR reaction. For this reason each set of primers were optimized for both annealing temperature and MgCl₂ concentration, within the ranges of 55-70°C, and 0.5-3.0 mM MgCl₂. Figure 3.1 illustrates an optimization for MgCl₂.

3.2.2 Identification of PCR product

Once optimized, each set of primers amplified cDNA of a single length, predicted by the original primer design. The reaction product was then digested with two separate restriction enzymes. The cut cDNA was run on an agarose gel to visualize and size. Uncut product was also run and Southern blotted. These bolts were then hybridized with full length cDNA labelled with ³²P and washed to high stringency. These two procedures confirmed the identity of the PCR products.
Figure 3.1 Optimisation of GDNFRα PCR primers

Lane 1 1kb DNA ladder, lane 2 1 mM MgCl₂, lane 3 1.5 mM MgCl₂, lane 4 3 mM MgCl₂, lane 5 H₂O negative control.

Figure 3.1b RT-PCR Products and Restriction Digested Fragments

(A) Lane one shows the PCR product produced by the ret primers. PvuI cuts ret at nucleotide number 133 producing fragments of 470 and 50 (too small to be detected on the agarose gel). (B) Lane one shows the PCR product produced by the GDNF primers. EcoRI cuts GDNF at nucleotide number 302 producing fragments of 170 and 165. (C) Lane one shows the PCR product produced by the GDNFRα primers. RSAII cuts GDNFRα at nucleotide number 1234 producing fragments of 300 and 120. (D) Lane one shows the PCR product produced by the RET/PTC2 primers. AccI cuts RET/PTC2 at nucleotide number 318 producing fragments of 265 and 132.
3.2.3 RT-PCR analysis of ret, GDNFRα, and GDNF expression

The organ distribution of the three genes during development and in the adult was examined by RT-PCR using total RNA extracted from the dissected organs. Tables contain the results of at least two repetitions for each sample and set of primers. The expression of β-actin was used as a positive control for both the RT and PCR reactions. Only samples positive for β-actin were subsequently used in screening for ret, GDNFRα, and GDNF.

3.2.3.1 Expression of ret by RT-PCR

Table 3.1 shows the expression of ret as examined by RT-PCR. These results largely agree with the published data for ret as assessed by in situ hybridisations and Northern blots (Pachnis et al., 1993). However, it is of note that, by RT-PCR, expression was detected in the developing lung, salivary gland, pancreas, and spleen. Expression within these organs has not previously been reported. Some E11 metanephroi were microdissected into metanephric mesenchyme and ureteric buds. These preparations demonstrated positive ret expression in the mesenchyme approximately 50% of the time (three batches of dissected mesenchymes, with three PCR reactions carried out on each batch). Figure 3.2 illustrates a reaction in which the renal mesenchyme was negative for ret. The ureteric bud was always found to be positive for ret expression. The liver was found to be negative for ret at all developmental stages, and so was used as a negative control for the contamination of tissues, or samples with spurious RNA or cDNA.

3.2.3.2 Expression of GDNFRα by RT-PCR

GDNFRα was expressed in all organs tested: brain, gut, heart, metanephros, liver, lung, pancreas, salivary gland, and spleen, as shown in Table 3.2. Expression was detected at all time points examined, from E10 to the adult. Interestingly, GDNFRα was expressed in both the epithelium and the mesenchyme of the ureteric bud (Figure 3.2). This pattern of expression far extended that seen for ret.

3.2.3.3 Expression of GDNF by RT-PCR

Table 3.3 summarises the expression of GDNF by RT-PCR. The expression of GDNF is similar to the expression of ret in the majority of tissues examined. Of particular interest were organs that develop by branching morphogenesis, such as the metanephros, lung, salivary gland, spleen, and pancreas (Figure 3.2). These organs were all positive for GDNF expression during development, with expression being restricted to the mesenchyme of the E11 metanephros (Figure 3.2). However, a few discrepancies were seen in the patterns of GDNF
Table 3.1 Expression of ret in mouse embryonic and adult organs by RT-PCR

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Each result was repeated twice. All samples used were positive for actin (data not shown).

Table 3.2 Expression of GDNFRα in mouse embryonic and adult organs by RT-PCR

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Each result was repeated twice. All samples used were positive for actin (data not shown).
Table 3.3 Expression of GDNF in mouse embryonic and adult organs by RT-PCR

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Each result was repeated twice. All samples used were positive for actin (data not shown).
Table 3.4 Expression of Ret, GDNF, and GDNFRα in renal cell lines

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Each result was repeated twice. All samples used were positive for actin (data not shown).
Figure 3.2 Expression of ret, GDNFRα, and GDNF in branching organs by RT-PCR

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M5, renal mesenchymal cell line; sg, salivary gland; ub, isolated ureteric bud; mm, isolated metanephric mesenchyme; H2O, water control.
and ret. In the adult heart there was no expression of GDNF, whereas ret was expressed in this organ. Conversely in the adult kidney, ret was no longer expressed although GDNF was detected.

3.2.3.4 Expression of ret, GDNFRα, and GDNF in metanephric cell lines by RT-PCR

The derivation of these cell lines has been previously described in (Woolf et al., 1995). Table 3.4 summarises the expression of the three genes in these cell lines. All the cell lines were found to be negative for ret and positive for GDNFRα. However, only 8 out of 11 were found to be positive for GDNF.

3.2.3.5 Summary of RT-PCR data

In summary, an investigation into the expression windows of the three genes, by RT-PCR, has expanded the previously published data. However, it was necessary to confirm this data with in situ hybridisations and to localise the transcripts to specific cellular populations. Of particular interest was the novel expression of the three genes in organs that develop through branching morphogenesis.

3.2.4 In situ hybridisation analysis of ret, GDNFRα, and GDNF expression during mouse embryogenesis

The probes used for the in situ hybridisations are described in Chapter 2. Sense probes demonstrated no hybridisation to any of the tissues under examination. Pictures of these have not been included in all cases, as the antisense probes demonstrated highly specific patterns of expression (Figure 3.3).

3.2.4.1 Expression during metanephros development

At E10 the Wolffian duct is juxtaposed to the intermediate mesoderm that will form the metanephric blastema, but the ureteric bud has not yet emerged. At this stage, all three transcripts were detected in the nephrogenic region with both ret and GDNFRα being expressed in the most caudal part of the Wolffian duct. The metanephric mesenchyme strongly expressed GDNF at E10, and also expressed a low level of GDNFRα (Figure 3.4). Although the RT-PCR data demonstrated ret expression in the renal mesenchyme in approximately 50% of the samples, this result was not confirmed in any of the embryos examined by in situ hybridisation, although this may be due to the higher sensitivity of PCR. The patterns of expression were maintained at E11.5. At this stage the ureteric bud has branched once. At E16 the expression of all three genes was restricted to the nephrogenic zone, the outer region of
the metanephros where active ureteric bud branching and nephron formation is still occurring. Ret expression was detected only in the terminal buds of the collecting duct branches. GDNFR α was also detected in the terminal buds and associated mesenchyme at lower levels. Unlike ret, GDNFRα was also expressed along the cortical collecting ducts. At all times, the expression of GDNF was restricted to the mesenchyme surrounding the branching tips. Under high power microscopy it was apparent that the expression of GDNF did not extend to the outermost edges of the organ where stem cells are thought to be located (Koseki et al., 1992).

3.2.4.2 Expression during lung development

mRNA of ret, GDNFRα, and GDNF had been detected in the lung by RT-PCR from E10.5 through to adulthood. However, no transcripts could be detected at E10, or E11.5 by in situ hybridisation, data not shown. At E13.5, developing lungs are large enough to be dissected out of the embryo and processed intact for whole mount in situ hybridisation. Using this method, all three genes were detected in E13.5 lung rudiments (Figure 3.5). The expression levels were relatively low when compared to those seen in the metanephros, requiring longer development times to visualise the reaction products. At E13.5 ret was detected throughout the developing lung epithelia and was not expressed in the mesenchyme. Expression of GDNFRα was detected in the epithelia, and associated mesenchyme, of the trachea and developing bronchi. However, it was absent from both cell types at the distal tips. Weak GDNF expression was observed in the mesenchyme juxtaposed to the lung throughout the organ. At E16.5, neither GDNFRα nor GDNF could be detected in the lungs. However, a punctate signal was detected for ret around the trachea. Although no secondary neuronal markers were used, the position of these cells coincides with the neurones of the adult lung (Figure 3.6 A).

3.2.4.3 Expression during salivary gland development

Ret has previously been detected in the adult salivary glands by Northern blots (Pachnis et al., 1993), however, its cellular localisation was unknown. E13.5 and E16.5 lower jaws were examined to assess the expression pattern of the three genes within the developing salivary gland. Ret expression was not detected in the epithelium or mesenchyme of the salivary gland, at either stage, by in situ hybridisation. The salivary gland has two large nerve ganglia at the base of the duct which develop into a rich nervous system both in vivo and in vitro (Coughlin, 1975); expression of ret was exclusively associated with these structures. Expression of GDNFRα was detected in the salivary gland mesenchyme at E13.5 and E16.5. GDNF was not detected within the organ at either time point (Figure 3.5).
Figure 3.3 Whole mount in situ hybridisation of E10.5 embryos

A

B

C

D
Figure 3.3 Whole mount in situ hybridisation of E10.5 embryos

(A) Ret staining is observed in the facioacoustic ganglion and the inferior complexes of the VIIth, IXth and Xth cranial ganglia. Expression is also detected in the caudal region of the Wolffian duct (arrows). (B) GDNFRα expression is also detected in the VIIth, IXth and Xth cranial ganglia, as well as the nephrogenic region (arrows). (C) GDNF expression in the pharyngeal clefts and the nephrogenic region (arrows). (D) A control embryo hybridised with a cocktail of ret, GDNFRα, and GDNF sense probes control.

ov = otic vesicle
Figure 3.4 continued
Figure 3.4 Expression of ret in the metanephros by in situ hybridisation

A, D, and G ret; B, E, and H GDNFRα; C, F, I, and J GDNF. A-C low power views of vibrotome-sectioned E10.5 embryos. Arrowheads in A-C indicate the caudal part of the Wolffian duct, and arrows in B and C indicate the caudal part of the lateral plate mesoderm; lb, limb bud; g, gut. D-J E16.5 organs. Ret was only detected in the terminal buds (tb), but some of the GDNFRα expression was seen in the cortical collecting ducts (arrowheads in E and curved arrow in H). The expression domain of GDNF did not extent to the very edge of the organ (open arros in I). J show an en face view, clearly demonstrating that GDNF was expressed by the mesenchyme closely associated with the terminal buds. Bars in A-C, 80 μm; D-F, 40 μm; and G-J, 20 μm.
Figure 3.5 Expression of ret, GDNFRa, and GDNF in the E13.5 lung and salivary gland, by in situ hybridisation
Figure 3.5 Expression of ret, GDNFRα, and GDNF in the E13.5 lung and salivary gland, by in situ hybridisation

(A and D) ret, (B and E) GDNFRα, and (C and F) GDNF. In the lung (A-C) ret was expressed throughout the epithelium including the distal buds (db). GDNFRα was detected in the proximal buds and the adjacent mesenchyme in this area (arrowheads in B), but not in the distal buds. GDNF was weakly expressed by the mesenchyme proximate to the ret-expressing epithelium, in all areas of the organ (arrowheads in C). In the salivary gland (D-F) ret was not expressed in the epithelium (e), but in two structures which were probably developing ganglia (g); GDNFRα was also absent from the epithelium, but was seen in the mesenchyme (arrowheads in E); while GDNF was absent from the organ. The magnification is the same in all micrographs; bar in A=40 μm.

3.2.4.4 Expression during organogenesis of the pancreas

The pancreas is another organ that develops through branching morphogenesis (Larson, 1993). At E11, during the very early stages of its development the expression of neither GDNF nor its receptors could be detected by in situ hybridisation. However, at E16.5, the majority of cells within the pancreas could be seen to be positive for ret (Figure 3.6) but not for GDNF, or GDNFRα.

3.2.4.5 Expression during organogenesis of the thymus

The thymus also develops by branching morphogenesis (Larson, 1993). Clear expression of GDNFRα was detected throughout the cells of the thymus at E16.5, but GDNF or ret were not detected (Figure 3.6).

3.2.4.6 Expression during development of the gastrointestinal tract

At E10, a population of ret positive cells were detected within the mesenchymal layer of the gastrointestinal tract. At later stages, these cells began to coalesce to give a punctate staining pattern. This confirms previous data reported by Pachnis et al. (1993), who described ret positive cells in the migratory pathway of the presumptive enteric neuroblasts, and in the myenteric ganglia of the gut. At E10, the expression pattern for GDNF and GDNFRα was restricted to the mesenchymal cells of the gastrointestinal tract. As the gut develops, from E10.5-16.5, it is covered by splanchnic mesoderm. This is composed mostly of loose mesenchymal cells, which will contribute to the formation of the connective tissue and smooth muscle layers. As development progressed, the expression of GDNFRα and GDNF was restricted to the outer mesenchymal layer of the gut and in later stages, in the outer smooth
muscle layer (Figure 3.7). These data correlate to that of a previous study reporting the expression of GDNF (Hellmich et al., 1996).

3.2.4.7 Expression in the developing limbs

At E11, the tissue of the limb buds was mostly negative for ret expression. However, a stream of individual ret positive cells could be identified. These cells were detected in both the hind and fore limb buds and appeared to be closely associated with a putative nerve fibre. No independent molecular markers were used in these experiments, however, the overall appearance and position of these ret positive cells suggests that they may be neural crest derived Schwann cells, which are known to migrate along nerve tracks in the developing limb (LeDouarin, 1997). At E10.5, expression of GDNF was localised to the posterior region of the limb buds, in the zone of polarizing activity (Vogel and Tickle, 1993). At no point did the limb buds show any expression of GDNFRα (Figure 3.8).
Figure 3.6 Expression of ret, GDNFRα, and GDNF in the E16.5 lung, salivary gland, thymus, and pancreas
Figure 3.6 Expression of ret, GDNFRα, and GDNF in the E16.5 lung, salivary gland, thymus, and pancreas

Punctate expression pattern of ret in the lung (A) and salivary gland (B). In the lung the ret positive cells were located around trachea (t) and main bronchiols (b). In the salivary gland the ret positive cells were located throughout the central and proximal areas of the organ. (C) Cortical expression of GDNFRα in the thymus (th). (D) Expression of ret in the pancreas (p); gut (g). The magnification is the same in all micrographs; bar in A=40 μm.
Figure 3.7 Expression of ret, GDNFRα, and GDNF in the E11 and E16.5 gut
Figure 3.7 Expression of ret, GDNFRα, and GDNF in the E11 and E16.5 gut

Expression of ret in the E11 (A, C, and D) and E16.5 (B, D, and F) gut. (A) Expression of ret is detected in the mesenchymal layer of the developing gut where the vagal neural crest are beginning to coalesce to form the ganglia of the gut. (B) By E16.5 ret expression is continued to be expressed in these ganglia. (C) Expression of GDNFRα is detected in the mesenchymal cells surrounding the gut epithelia. (D) At E16.5 GDNFRα is restricted to the outer smooth muscle layer. (E and F) Expression of GDNF is detected in the same pattern as GDNFRα, however, the signal is of a greater intensity. The magnification is the same in A, B, C, and E; bar in A=20 μm. Bar in D and E=40 μm.
Figure 3.8 Expression of ret, GDNFRα, and GDNF in the E10 limb buds
Figure 3.8 Expression of ret, GDNFRα, and GDNF in the E10 limb buds

Expression of ret (A and B) and GDNF (C). (A and B) Ret expression was restricted to a small population of cells that appeared to be migrating along a possible nerve tract (arrows). (C) GDNF expression was detected in the posterior region of the limb buds (lb), in the zone of polarizing activity. (g) gut; (nt) neural tube. The magnification in A and C is the same, bar in A=80 μm. Bar in B=20 μm.

3.2.4.8 Expression in the ectodermal placodes and their neurogenic derivatives

Neural crest cells migrating from rhombomere 4 into the second branchial arch have previously been reported to express ret, with the second branchial arch and more posterior arches also showing positive expression (Pachnis et al., 1993). Preliminary data for the expression of GDNFRα and GDNF in cranial embryonic structures are presented in Appendix 1. The surface ectoderm gives rise to the inferior ganglia of the IXth and Xth cranial nerve ganglion complexes. These ganglia were positive for ret and GDNFRα at E11 (Figure 3.3).

GDNF expression was clearly detected in the epithelial thickenings of the olfactory placode and also in the endoderm of the foregut roof at E10. At E11, there was a substantial invaginated cavity, the epithelial lining of which expressed GDNF. The mesenchyme associated with the placodal epithelia expressed ret and very low levels of GDNFRα (Figure 3.9).

3.2.4.9 Expression in the central nervous system

During the period of embryogenesis examined, ret, GDNFRα, and GDNF were all expressed within the developing CNS. More specifically, at E10, ret expression was detected in the ventrolateral compartment of the spinal cord, where the motor neurons are differentiating. In the DRGs of these embryos, intense punctate signal was observed, however, this signal was not detected in the emerging peripheral spinal nerves. This expression pattern agrees with previous studies where it was suggested to represent a sub population of cells within the DRG (Pachnis et al., 1993). The expression was absent from the glial cells of the ganglion and spinal nerve. GDNFRα and GDNF were also detected in the DRG, although unlike ret, transcripts were apparent throughout the cells of these ganglia (Figure 3.10).

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Figure 3.9 Expression of ret, GDNFRα, and GDNF in the E10 olfactory placode
Figure 3.9 Expression of ret, GDNFRα, and GDNF in the E10 olfactory placode

(A) Expression of ret in the mesenchymal cells around the placodal epithelia. (B) Expression of GDNFRα throughout the mesenchymal tissue. (C) Expression of GDNF in the olfactory epithelium. Arrows indicate olfactory invagination. The magnification in A and C is the same, bar in A=80 μm. Bar in B=40 μm.
Figure 3.10 Expression of ret, GDNFRα, and GDNF in the E11 CNS

A

drg

nt

B

drg

C

105
Figure 3.10 Expression of ret, GDNFRα, and GDNF in the E11 CNS

(A) Ret expression is observed in the motor neuron columns at the ventral part of the spinal cord and in individual cells of the dorsal root ganglia (drg). GDNFRα (B) and GDNF (C) are expressed in similar patterns. In the neural tube (nt) expression is located dorsally of the motor neuron columns and is expression throughout the cells of the dorsal root ganglia. The magnification in A and B is the same, bar in A=80 μm. Bar in C=40 μm.

3.2.5 Protein expression in the metanephros, lung, and salivary gland

The anti ret antibodies used in this study were described in Chapter 2. Three of the antibodies were polyclonal, SC1, B1, and B2, with the A1, being a cocktail of three hybridoma supernatants.

SC1 gave no binding on either Western blots or on whole mount immunohistochemistry and so will not be discussed further.

On Western blots, B1 bound to many proteins of various sizes and thus demonstrated little specificity. On whole mount preparations, B1 gave apical, punctate binding on metanephroi, lungs, and salivary glands. This correlates to the immunoreactivity described by Liu et al. in the developing metanephros (Liu et al., 1996). However, these investigators also reported immunoreactivity in the developing nephron epithelia. As described above, it has been conclusively shown, by in situ hybridisation, that ret expression is restricted to the ureteric bud and its derivatives and is not expressed by the metanephric mesenchyme. For this reason it was assumed that the binding by the B1 antibody was nonspecific.

On Western blots, both B2 and A1 bound to many proteins and so were highly nonspecific under these conditions. However, on whole mount preparations the immunoreactivity patterns of both antibodies showed basolateral binding to the epithelium of the ureteric bud, lung, and salivary gland. The controls for A1, with no primary antibody, gave exactly the same staining pattern. This suggests that the immunoreactivity was due to nonspecific binding of the secondary antibody. The primary antibody could possibly have been used with an alternative secondary antibody, although due to time restrictions these optimisations were not undertaken.

Controls for the B2 antibody gave a very low background, suggesting that B2 was specifically binding to the ret protein. Figure 3.11 shows the immunoreactivity of the B2 antibody on the metanephros, lung, and salivary gland. All three organs demonstrate basolateral localisation of the epithelium. From the in situ hybridisation results described above, the expression of ret in the salivary gland was restricted to the large nerve ganglia at the base of the epithelial bud and was not detected in the epithelium itself. Taking these results into consideration, it appears that the B2 antibody is also binding nonspecifically.
In summary, of the four antibodies used, none gave a convincingly specific localisation of ret, which corresponded to the results from the in situ hybridisation described above. After due consideration the results from the immunohistochemistry were deemed inconclusive.
The B2 antibody demonstrated basolateral localisation of the ret protein in the E12.5 metanephros (A), the E12.5 lung (C) and the E13.5 salivary gland (E) (arrowheads). (B, D, and F) Controls containing no primary antibody. The magnification in A to D was the same. Bar in A=20 μm. The magnification in E and F was the same. Bar in E=40 μm.
3.3 Discussion

The spatial and temporal expression patterns of ret, GDNFR\textalpha{}, and GDNF have been assessed by RT-PCR and whole mount in situ hybridisation. The results described above demonstrate coordinated expression of ret, GDNFR\textalpha{}, and GDNF in many areas, suggesting roles for this pathway during the interaction of epithelia and mesenchyme during organogenesis. In addition, there is a probable role for this pathway during migration, differentiation, or survival of various neuronal cell lineages.

Using RT-PCR analysis, GDNFR\textalpha{} appeared to be widely expressed during embryogenesis, suggesting that it may be ubiquitously involved in many receptor-ligand interactions. The data from the in situ hybridisations gave a much more specific pattern of expression. The inconsistency between these two results probably reflects the high sensitivity of RT-PCR, which enabling amplification from very low copy numbers of mRNA. It is debatable whether genes expressed at such a low copy number are functionally relevant.

3.3.1 Ret, GDNFR\textalpha{}, and GDNF are expressed in contrasting expression patterns in different developing organs

In situ hybridisation revealed that, at E10.5, shortly before the metanephric kidney primordia can be recognised, ret transcripts were already present in the most caudal part of the Wolffian duct. Hence, ret is expressed in the Wolffian duct prior to ureteric bud outgrowth. GDNFR\textalpha{} followed a similar expression pattern to that of ret. However, it was also detected, at lower levels, in the most caudal part of the lateral plate mesoderm, the presumptive metanephric blastema. GDNF transcripts were present in the presumptive metanephric blastema only. These data support the hypothesis that the ret-GDNF signalling pathway is crucial at the inception of the metanephros, with GDNF being one of the mesenchymally derived factors which influences the growth of the ureteric bud.

At later stages, expression of ret and GDNF was detected in the nephrogenic zone, suggestive of a continuous role in metanephric development. A few organs were left to 'overdevelop' in order to visualise any low level expression in other cell types. Under these conditions it was confirmed that ret expression is exclusively expressed in the tips of the ureteric bud with GDNF expression being restricted to the mesenchyme. These results unequivocally demonstrate the expression patterns of ret and GDNF. The amplification of ret from the mesenchyme by RT-PCR is therefore, probably due to small amounts of contaminating ureteric bud or Wolffian duct tissue in the mesenchyme preparations. Previous reports have demonstrated the presence of ret in developing nephrons by immunohistochemistry.
(Coughlin, 1975). It has been irrefutably demonstrated here that ret is not expressed by the mesenchyme or its derivatives, suggesting that these data are probably due to non-specific binding of the antibody.

GDNFRα was also expressed in the nephrogenic zone with expression extending into the cortical collecting ducts. As well as expression in the ureteric bud, lower levels of GDNFRα were found in the mesenchyme closely associated with the tips of the ureteric bud, at E11 and E16.5. This suggests that GDNFRα does not only function to facilitate the binding of GDNF to ret, by its coexpression within the ureteric bud, but may also sequester GDNF within the mesenchyme that is secreting it. This in turn may act to maintain a high concentration of GDNF adjacent to the tissue expressing the receptor, as well as acting to prevent GDNF from diffusing throughout the nephrogenic region.

GDNF was only expressed by 8 out of 11 renal mesenchymally derived cell lines (Woolf et al., 1995). From the in situ hybridisation it was seen that GDNF expression did not extend to the outermost region of the E16.5 metanephric mesenchyme, where nephrogenic stem cells are located (Bard et al., 1994). It is possible that these cells, negative for GDNF, are cell lines derived from these renal stem cells.

In the lung at E10 and E11.5, in situ hybridisation did not detect transcripts for any of the three genes, suggesting that this pathway is not critical for early lung development. At E13.5, all three genes could be detected by in situ hybridisation. The expression pattern at this stage, was similar to that seen in the metanephros, but with some notable differences. All transcripts were expressed at lower levels than in the metanephros, requiring longer development times. Ret was detected throughout the epithelium, and was not restricted to the developing tips, as seen in the ureteric bud. Contrary to the expression seen in the metanephros, GDNFRα was absent from the distal tips, but was detected in the more proximal epithelia and associated mesenchyme. As in the metanephros, GDNF expression was restricted to the mesenchymal component associated with the epithelia. However in the lung, GDNF was not restricted to the cells surrounding the branching tips, but was expressed in the mesenchyme adjacent to the epithelia throughout the developing organ.

As discussed in Chapter 1, the mesenchyme has an instructive influence on the development of the epithelia (Grobstein, 1967). Grobstein originally suggested that there are mesenchymal specific and mesenchymal common factors working together to orchestrate these influences. However, it has been demonstrated here that factors which are common to the metanephros and the lung can be expressed in both differing spatial and temporal patterns, and at different concentrations. Thus, Grobstein's original hypothesis can be expanded to include the idea that it is both the spatial and temporal expression patterns of biological factors and
their receptors, along with their individual concentration, that defines the influence of a pathway on a developing organ.

If this is indeed true, one would expect the recombination of ureteric bud epithelium with lung mesenchyme to produce a "lung-typic" epithelial branch pattern. This has recently been demonstrated by Kispert et al., in an elegant recombination experiment where a branch pattern typical of the lung was produced in ureteric bud epithelia (Kispert et al., 1996). From the in situ hybridisation results it appears that the level of GDNF in the lung is lower than that detected in the metanephros. However, this lower level of GDNF can sustain development of the ureteric bud for several reasons. Firstly, the lung mesenchyme has been reported to be more tightly packed (Lawson, 1974). This may help to keep the local concentration of secreted GDNF relatively high by restricting its diffusion. Secondly, the expression of GDNF in the lung is located in the mesenchymal cells immediately juxtaposed to the epithelia, thus, maximising the concentration of GDNF at the epithelial-mesenchymal interface. Thirdly, as already discussed in the Chapter 1, there may be low and high affinity GDNFRα isoforms. Perhaps the isoform expressed in the lung exhibits a higher affinity for GDNF than that expressed in the metanephros, thus binding a lower GDNF and activating ret at a lower concentration.

In the salivary gland, ret and GDNF were essentially absent from the development of the epithelial and mesenchymal cell lineages. The presence of GDNFRα within the mesenchyme suggests that it may be working in conjunction with another receptor.

3.3.2 Expression in the gastrointestinal tract

Ret transcripts are detected caudal to the posterior branchial arches and in anterior regions of the gut consistent with the anterior-posterior migration of enteric neural crest cells. This data is consistent with previous reports in the mouse and chicken (Pachnis et al., 1993; Robertson and Mason, 1995; Schuchardt et al., 1996). GDNFRα was more ubiquitously expressed in all the cells of the gut mesenchyme. As with the metanephros and lung, discussed above, the expression of GDNFRα extended that seen for ret. This suggests that GDNFRα is again working to prevent the diffusion of the secreted GDNF. The early expression of GDNF in the mesodermal mesenchyme surrounding the gut endoderm and its continued expression in the outer smooth muscle layers of the gut, throughout the entire length of the digestive tube, suggests that it plays an important role in the development and subsequent function of the mature gut. Examination of older embryos, at E16.5, demonstrated that ret is expressed in cells of both the submucosal and myenteric plexi, as previously described (Pachnis et al., 1993; Robertson and Mason, 1995). Null mutations of either ret or GDNF causes a failure of the migration, or survival of the vagal neural crest (Schuchardt et al., 1994; Sanchez et al., 1996;
The expression of GDNF along the entire length of the gut suggests that it is not functioning as a directional cue for these neural crest, but acting as survival factor for vagal neural crest cells, and subsequently the nerves of the submucous and myenteric plexi.

3.3.3 Expression in the developing limb buds

GDNF was found to be expressed in the developing hind and fore limb buds. It has previously been suggested that this may correlate with GDNF having a target derived role in motor neuron development (Lindsay, 1995). However, neither ret nor GDNFRα were detected in the developing nerves of the limb buds, although a subset of cells that appeared to be migrating along the nerve tracts were positive for ret. These cells are probably neural crest derived Schwann cells, which are known to have this migratory phenotype during limb development (LeDouarin, 1997). Although no GDNFRα was detected, GDNF may be acting on the differentiation or survival of these cells. The use of secondary markers for both the nerve tracts and Schwann cells could be used to confirm the identity of these cells.

3.3.4 Expression in the ectodermal placodes and their neurogenic derivatives

Neurogenic placodes are focal ectodermal thickenings that give rise to the sensory neurons, and in some cases, the receptor cells of vertebrate sensory systems. There are at present, no markers for undifferentiated placodal epithelia. By invagination and or delamination, placodal epithelia forms sensory neuroblasts, sensory epithelia and in some cases, migratory receptor primordia (Webb and Noden, 1993). The expression of the ret-GDNF pathway was detected in the olfactory placodes which give rise to the olfactory nerves.

The expression of GDNFRα and GDNF in the DRGs is consistent with the expression of ret in a subset of neurons within these structures. Together these results strengthen the hypothesis that the ret-GDNF pathway is involved in the proliferation, migration, differentiation, or survival of a variety of neuronal cell lineages.

3.4 Further work

Expression of the ret protein in the enteric nervous system and metanephrors has previously been reported (Tsuzuki et al., 1995). However, the protein localisation for either GDNFRα or GDNF has not been investigated. A hypothesis has been put forward above that
GDNF is sequestered by the cells expressing GDNFRα. Immunohistochemical studies could demonstrate the localisation of both proteins and show whether this is indeed the case.

As mentioned in Chapter 1, it has been hypothesised by Jing et al (1996) that there may be different GDNFRα isoforms with varying affinity for GDNF. The ribo-probe used in this study consisted of the 3' end of the cDNA, which contains the membrane binding domain. It is unlikely that this probe could differentiate between any such isoforms, as the affinity for GDNF probably lies in the extracellular domain. However, cloning of the different cDNAs, followed by their use as riboprobes, may yield differing expression patterns for the isotopes, thus explaining the high dependence of the metanephros and enteric nervous systems on high levels of GDNF (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996).
4. The effect of GDNF on the development of the metanephros, lung, and salivary gland in vitro

4.1 Introduction

During organogenesis of the metanephros, lung, and salivary gland, the configuration of branching tubules, with swollen end buds, in contact with a mesenchymal tissue ensues normal development. Initially, an epithelial outgrowth surrounded by undifferentiated mesenchyme undergoes mutual induction. These elements depend upon each other for survival, growth, and differentiation (Grobstein, 1967), resulting in dichotomous epithelial branching. The high level of branching increases the surface area of the epithelia, with each individual organ having its own specific branch pattern (Spooner et al., 1986).

Studies in the 1950s by Grobstein (Grobstein, 1953) demonstrated that developing organs can be grown in vitro. This allowed the manipulation of specific molecular pathways and dissection of their functional role(s). More recently, genetic manipulation of genes has allowed the production of mice carrying genetically engineered alleles. Although this technology is a powerful tool for investigating the role of certain molecules in development, it is often difficult to decipher whether the phenotype is of primary or secondary origin. For example, pulmonary hypoplasia can be caused by physical restrictions on the developing lung, such as insufficient quantities of amniotic fluid in Potter’s syndrome, or simply a lack of space for the lungs to grow as seen in some thoracic skeletal dysplasias (Page and Stocker, 1982). Phenotypes range from normal development to a very severe embryonic lethality. Ret or GDNF null mutant mice die soon after birth with complete agenesis or severe dysgenesis of the metanephros (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996). In a situation such as this it appears that the genes are involved in the inception of the metanephros, but the transgenic model does not allow one to study the role of the genes in further development. In contrast, organ culture permits the manipulation of a pathway at later stages of development, in isolation from confounding developmental lesions so that roles in later development can be assessed. It also allows the investigation into dose related responses, a question not easily addressed with transgenic technology. For these reasons, in vitro organ culture was used for the investigation into the role(s) of the ret-GDNF signalling pathway in branching morphogenesis.

In this study, the developing metanephros, lung, and salivary gland were manipulated in vitro. The development of each of these organs has already been described in Chapter 1 and
so shall not be detailed again here. One salient difference between these organs is the process of branching. The metanephros develops by the budding of the ureteric bud, whereas the salivary gland branches by the formation of clefts in the epithelia, with the lung being thought to do both. The different final branch pattern in the three organs is probably reflects their various growth strategies (Spooner et al., 1986). Due to the different cellular mechanisms used during the branching morphogenesis of the epithelia, one may expect to see differential gene expression patterns, and this has been shown to be true for the ret-GDNF signalling system, as described in Chapter 3. A further difference between the three organs is the fate of the mesenchyme. In the majority of epithelial organs, including the lung and salivary gland, it is the epithelial component which will form the tissue of primary function in the adult organ, with the mesenchyme differentiating to form essential supporting tissues such as the stroma and vasculature. In the metanephros, however, both the ureteric bud and renal mesenchyme form important functional epithelial components in the adult organ: the ureteric bud gives rise to the collecting duct system and the metanephric mesenchyme gives rise to the nephrons (Bard et al., 1994; Patterson and Dressler, 1994), with a small contribution of mesenchymal cells into the collecting duct system (Qiao et al., 1995).

In this study, it has been shown that GDNF acts in a dose dependent manner on epithelial morphogenesis in the metanephros; addition of exogenous GDNF in vitro causes an increase in evagination, growth, and branching of the ureteric bud. In contrast GDNF does not affect the development of the lung or salivary gland.

4.2 Materials and methods

The organ culture methodology has been described in the methods section of Chapter 2. Organ rudiments were isolated from embryos by microdissection at E11 (metanephros), late E10 (lung), or late E12 (salivary gland). Metanephroi were usually dissected free of the Wolffian duct, however, in one experiment the duct was left intact to examine the effect of various treatments on this epithelium. Rudiments were randomly assigned to control or treatment groups. Two or three separate experiments were performed for each organ and treatment, with 5-20 rudiments in each treatment group. The morphology was documented with photomicrographs using an inverted microscope. The treatments were:

i) growth in 50, 100, or 500 ng ml\(^{-1}\) GDNF

ii) 10 or 50 ng ml\(^{-1}\) blocking antibody to GDNF

iii) 50 ng ml\(^{-1}\) normal goat IgG

iv) the final controls had nothing added.
Lung cultures were grown for 4 days, salivary gland cultures for 3 days and metanephroi cultures for 2 days. After this time the organs were fixed for 30 minutes in 4 \% PFA, before being stained with anti laminin antibodies. Laminin is a major component of the basement membrane of developing epithelia. Within the kidney this includes both the branches of the ureteric bud as well as the developing nephrons. Use of this antibody allowed the identification of several different stages of nephron formation, including vesicles, comma-shaped and S-shaped bodies, all of these stages were grouped together as nephron forming units (NFUs). Subsequent analysis used the confocal microscope to allow quantification of the end buds and NFUs.

4.3 Results

4.3.1 GDNF promotes epithelial development during nephrogenesis

The effect of exogenous GDNF on kidney, lung, and salivary gland rudiments grown in culture was studied, with particular reference to the development of the epithelia.

4.3.1.1 Addition of exogenous GDNF to whole metanephroi in culture

The branching morphogenesis of the developing metanephros was not responsive to low doses of exogenous GDNF. When E11.5 metanephroi were cultured in 10-50 ng ml⁻¹ GDNF there was no visible effect. However, when cultured in 100-500 ng ml⁻¹ GDNF there was a dramatic increase in ureteric bud development. The most profound effect was the formation of supernumerary ectopic buds which sprouted from the base of the main ureteric bud (Figure 4.1). This occurred in 11 out of 22 cultures. In one experiment the Wolffian duct was left attached to the metanephroi. In this case ectopic buds were seen developing along the entire length of the caudal region of the Wolffian duct. Of particular not these supernumerary buds did not always grow towards the mesenchyme. The latter being always the case in the controls (only occurring in 3 out of 30 cultures).

As well as the development of supernumerary ureteric buds, GDNF caused a significant increase in the number of branch tips and NFUs (see Table 4.1 and Figure 4.2). Once the number of NFUs in the GDNF treated cultures had been normalised for the increase in epithelial growth, there was no significant difference between the number of NFUs per end bud in the controls and experimental organs.
4.3.1.2 Addition of blocking GDNF antibodies to whole metanephroi in culture

When embryonic metanephroi were cultured in media containing a blocking antibody to GDNF, their morphogenesis was severely impaired in a dose dependent manner. 10 μg ml⁻¹ of blocking antibody caused minor effects on the epithelial development, but 50 μg ml⁻¹ caused a significant reduction in the number of epithelial end buds. Some cultures failed to produce any secondary branching at all, and were unable to induce the surrounding mesenchyme to form condensates (Figure 4.2). The overall number of NFUs was reduced in the presence of blocking antibody. However, once normalised to the reduction in end bud numbers it was found to not be significantly different (Table 4.1). The specificity of the antibody was shown by the ability of pre-incubation of the antibody with GDNF to neutralise these effects.

The effects on the kidney were only seen at relatively high concentrations of either growth factor or antibody, shown in the dose dependent response peaking at 100 ng ml⁻¹ GDNF and 50 μg ml⁻¹ blocking antibody to GDNF. To exclude the possibility that any of the effects on the epithelia were due to toxicity, control experiments were performed using equivalent concentrations of normal IgG. There was no significant differences in the cultures grown in 100 ng ml⁻¹ GDNF to those grown in 500 ng ml⁻¹ GDNF. At this higher concentration of growth factor, and in the culture grown with IgG, no toxic effects, such as areas of necrosis, were seen.

4.3.2 GDNF does not affect branching morphogenesis of lung or salivary gland rudiments in culture

The expression pattern of ret, GDNFRα, and GDNF in the lung (as described in Chapter 3) suggests that this signalling system may be involved in the development of the lung. However, isolation and culture of early lung rudiments in media containing GDNF, or blocking antibodies to GDNF, for four days did not grossly affect its development. The size and shape of the organ was similar to the controls with equivalent numbers of epithelial branches (Figure 4.1). To confirm that there were no minor lesions in the development of the lung rudiments, cultured organs were fixed and embedded in wax for sectioning, followed by staining with haematoxylin and eosin. After close inspection of the experimental and control cultures it was concluded that there were no differences between them (data not shown).

Although the three genes are expressed in the salivary gland, as described in Chapter 3, this expression is thought to be restricted to the ganglia of this organ. The addition of GDNF or blocking antibodies to GDNF did not affect the development of the salivary gland over a three day period, in vitro. The organs underwent growth and branching morphogenesis with no visible differences between the control and experimental groups(Figure 4.1). Although this
result was not unexpected, it functions as a control for the normal development of epithelial organs the culture system employed. It can therefore be concluded that the phenotype of the cultured metanephroi was solely due to the disruption of the ret-GDNF signalling pathway.

Table 4.1 The effect of GDNF and blocking antibodies against GDNF on ureteric bud branching

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Number of terminal buds*</th>
<th>NFU/end bud*ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (30)</td>
<td>6.5 (5 - 9)</td>
<td>0.63 (0.4 - 2.0)</td>
</tr>
<tr>
<td>GDNF 100 ng/ml-1 (28)</td>
<td>11.5 (11 - 14)*</td>
<td>1.81 (0.5 - 3.4)</td>
</tr>
<tr>
<td>Anti-GDNF 50 µg/ml-1 (21)</td>
<td>4.0 (3 - 6)*</td>
<td>1.00 (0.7 - 1.8)</td>
</tr>
</tbody>
</table>

(a) Data are expressed as median (25 % - 75 % percentiles).
(B) Nephron forming units
(*) p<0.05 vs control

Table 4.2 Occurrence of supernumerary ureteric buds in cultured metanephroi

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wolffian duct absent*</th>
<th>Wolffian duct present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/22</td>
<td>3/8</td>
</tr>
<tr>
<td>GDNF 100 ng ml-1</td>
<td>11/21</td>
<td>7/7</td>
</tr>
<tr>
<td>Anti-GDNF 50 µg/ml-1</td>
<td>0/11</td>
<td>3/10</td>
</tr>
</tbody>
</table>

(a) See text for details

Figure 4.1 Confocal images of cultured metanephroi

Metanephroi were treated to show branching and nephron formation and were visualised using the confocal microscope. (A) Control. (B, D, and E) GDNF at 100 ng ml-1. (C) anti-GDNF at 50 ng ml-1. (D and E) Ectopic buds (eb) can be seen growing from the base of the ureteric duct (D) and Wolffian duct (E). *A, main branch of the ureteric bud; tb, examples of terminal buds; n, examples of nephrons; wd, Wolffian duct. Bar in A=100 µm, all images are to the same scale. A and B are composites of 3 and 4 images, respectively, and each image is a reconstruction of 4 optical sections taken at 5 µm intervals.
Figure 4.1 Confocal images of cultured metanephroi
Cultured salivary glands (A-D) and lungs (E-H). (A) Late E12 salivary gland, 0 hours in culture. (E) late E11 lung, 0 hours in culture. (B and F) Control cultures at 2 days. (C and G) GDNF treated cultures at 2 days. (D and H) Anti-GDNF antibody treated cultures at 2 days. (S) sublingual gland; (sm) submandibular gland; (g) ganglia. The sublingual gland was absent in D. The magnification is the same in all micrographs and the bar in A=125 µm.

4.4 Discussion

The results presented here demonstrate that GDNF modulates epithelial branching, in vitro, during mouse metanephros development, but does not affect the development of the lung or salivary gland. Exogenous GDNF causes both the ectopic growth of supernumerary epithelial buds from along the length of the Wolffian duct and the base of the ureteric bud. In addition GDNF also causes an increase in the growth and branching of the ureteric bud derived structures. The effect is specific to epithelia expressing ret as GDNF did not effect the growth and development of the salivary gland.

GDNF significantly enhanced both branching of the ureteric bud and nephron formation, while blocking antibodies had the converse affect. These data strongly support the hypothesis of Pichel et al. (1996) that the ret-GDNF signalling pathway is essential, not only for the initial outgrowth of the ureteric bud, as suggested by the ret and GDNF null mutant mice (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996), but also throughout the development of the metanephros. In 1996 Vega et al. demonstrated that conditioned medium from the B49 cell line could increase branching morphogenesis of metanephroi grown in culture for four days (Vega et al., 1996). It has been demonstrated here that these effects can be achieved by the addition of GDNF alone, thus suggesting that the active component secreted by B49 cells is GDNF. GDNF-treated metanephroi contained significantly more NFUs than the controls. However, once corrected for the increase in ureteric bud development, the actual number of NFUs per terminal bud was the same under all conditions. This data quantitatively demonstrates that the number of nephrons within the developing metanephros is related to the development of the ureteric bud. However, this relation is not absolute, as nephrons can develop in the absence of normal ureteric bud growth and development (Young et al., 1991; Kreidberg et al., 1996).

In addition to the effects on the primary ureteric bud, GDNF stimulates the formation of supernumerary, ectopic buds from both the stalk of the primary bud and from points along the Wolffian duct. These ectopic buds often grew away from the metanephric blastema, a phenomenon not previously reported. From these data it appears that GDNF is working as a chemotactic factor directing ureteric bud growth. In the culture the normal cue of
mesenchymally derived GDNF is saturated by exogenous GDNF. This results in the ureteric bud growing away from the metanephric blastema. These data also demonstrate that the entire caudal region of the Wolffian duct is capable of ureteric bud outgrowth. The local concentration of GDNF determines the position and direction of bud formation, rather than contact with the mesenchymal cells themselves, as previously suggested (Saxen and Lehtonen, 1978).

It has been postulated in the previous chapter that GDNFRα, expressed by the mesenchyme, may be acting to sequester the secreted GDNF. This capturing of GDNF would fulfill two functions. Firstly, it would prevent the diffusion of GDNF through the lateral plate mesoderm, thus insuring the outgrowth of a single ureteric bud into the metanephric blastema. As the metanephros develops, it moves rostrally away from the Wolffian duct, with the nephrogenic zone being located at the proximal edges of the organ. This produces a distance between the cells secreting GDNF and the responsive Wolffian duct. GDNF is captured in the nephrogenic zone by GDNFRα and could therefore, not diffuse over this distance. Thus preventing the production of secondary ectopic buds in vivo. Secondly, the capturing of GDNF within the mesenchyme would help to maintain the relatively high level of GDNF necessary to elicit the activation of ret. Low levels of GDNF which are not captured within the nephrogenic zone by GDNFRα would not stimulate ureteric bud outgrowth as the concentration is not high enough.

It has previously been suggested that the absence or rudimentary morphology of the ureteric bud in the ret null mutant mouse could be due to a delay in its interaction with the metanephric mesenchyme (Schuchardt et al., 1996). The present study disproves this hypothesis, as it has been shown that the duct retains its ability to respond to GDNF, in vitro. This suggests that it is the inability of the duct to respond to GDNF that causes the phenotype of the ret null mutant mice (Schuchardt et al., 1994). It has also been demonstrated that the local concentration of GDNF needed to stimulate ureteric bud outgrowth is relatively high. This finding is in agreement with data from the GDNF null mutant mice, where the homozygous mutants have an increased incidence of unilateral renal malformations as compared to their heterozygous litter-mates (Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996): the heterozygous mice are producing some, but not sufficient concentrations of GDNF to sustain normal metanephric development.

It is likely that additional soluble factors produced by the mesenchyme are necessary for complete ureteric bud development. Davies et al. demonstrated that the branching and elongation of ureteric bud branches are under separate control (Davies et al., 1995). Data presented here suggest that GDNF is the signal which initiates the evagination of the ureteric bud from the Wolffian duct and subsequently acts on the formation of new branch-points at the
growing ureteric bud tips. Once formed, elongation and differentiation of the new branches may be dependent on other factors, perhaps including HGF (Woolf et al., 1995). This explains the phenomenon that isolated buds, cultured in collagen gels in the presence of GDNF, do not grow and branch. However, these cultures do form "bumps", which are possibly very immature, potential, branches (Towers et al., 1997). This is a similar phenotype to the MDCK cells transfected with ret (see Chapter 5).

A further aim of the current study is to address the question of whether the ret-GDNF signalling system has a generic role in branching morphogenesis. The phenotype of the null mutant mice for ret and GDNF suggests that this pathway is specific to the metanephros (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996). The expression pattern of the pathway in the developing lung is similar to that seen in the metanephros, implicating this signalling pathway in lung development (see Chapter 3). However, the lack of phenotype in both the cultures described here and in the GDNF null mutant mice (Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996) suggests that GDNF is not one of the rate limiting steps during lung development.

Ret, GDNFRα, and GDNF are essentially absent from the developing salivary gland and as expected, these organs developed normally in culture.

The results reported here clarify the function of the ret-GDNF signalling pathway in the development of the metanephros, but leave the role of this pathway in the development of the lung open to further investigation.

4.5 Further work

The results discussed above confirm the importance of the ret-GDNF signalling system in the development of the metanephros but question the role of this pathway in development of other branching organs.

To further investigate the role of the ret-GDNF pathway in the development of the lung one could use the model of the null mutant mice. Firstly, sectioning of homozygous mutant neonatal lungs and their normal littermates could ascertain whether there are abnormalities in the development of this organ. Removal and culture of the lungs at an early stage, as described in the above chapter, would allow them to develop without external influences. If lung development had been affected by the null mutation it would manifest itself in vitro, where it could be manipulated and assessed further.

It has been suggested that GDNFRα is of vital importance for the binding of GDNF to ret (Treanor et al., 1996; Jing et al., 1996). The existence of as yet unknown receptors with different affinity for either GDNF or ret may be confusing the results of this project and of the
phenotype of the null mutant mice (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996). Mice carrying a null mutation in the GDNFRα gene may help to elucidate the importance of the ret-GDNF pathway in branching organs other than the kidney. Results from Chapter 3 suggest that GDNFRα is expressed in the majority of developing organs. For this reason clear results may only be obtained if the gene is disrupted in an organ specific manner. For example, the surfactant protein C promoter has been used to target gene disruptions to the lung (Zhou et al., 1996).

Ret is expressed in the ganglia of both the lung and salivary gland (Chapter 3). Ret is also known to be expressed by developing parasympathetic nerves (Pachnis et al., 1993). The distribution of axon bundles can be visualised by staining supravitaly with methylene blue (Coughlin, 1975). One would hypothesise that there would be some disruption in their development within the lung or salivary gland, when cultured under the conditions described above.
5. Transfection of the normal and oncogenic forms of ret into three renal cell lines

5.1 Introduction

The expression pattern of the ret-GDNF signalling pathway, as described in Chapter 3, along with data from the null mutant mice studies for ret and GDNF (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996), suggest that the ret-GDNF signalling pathway is involved in the growth and development of the metanephros. It has been demonstrated in Chapter 4, that the in vitro effect of GDNF, on metanephric development, is to stimulate ureteric bud development. This suggesting that a putative role for GDNF is as a mediator of branching morphogenesis and/or growth of the ureteric bud. GDNF has also been shown to act as a neurotrophic factor, aiding the survival of both dopaminergic and motoneurones (Lin et al., 1994; Henderson et al., 1994) with the null mutant mice for either ret or GDNF exhibiting neuronal loss within the enteric nervous system.

Despite the fundamental involvement of GDNF in various biological phenomena the precise function(s) of the ret-GDNF pathway remain undetermined. It is therefore, important to examine the effect of GDNF in isolation and assess its function(s) in individual cell types. For this reason a full length cDNA and oncogene for ret (RET/PTC2) were subcloned into the expression vector pCI-neo. This vector was then stably transfected into M5, P1, and MDCK cell lines. RET/PTC2 is a recombination of the RET gene, in which the ret tyrosine kinase domain has been combined with part of the R1α regulatory subunit of protein kinase A. RET/PTC2 was originally isolated from a papillary thyroid carcinoma (Bongarzone et al., 1993). When this experiment was devised the ligand for ret was unknown. To allow investigations into the function of the activated form of ret, a constitutively phosphorylated mutant (RET/PTC2) was used (Bongarzone et al., 1993). However, this consideration was made redundant with discovery in 1996, that GDNF was a ligand for ret (Trupp et al., 1996; Durbec et al., 1996).

The M5 cell line is a mouse embryonic renal mesenchymal cell line (Woolf et al., 1995). It is relatively undifferentiated and is assumed to be derived from uninduced metanephric mesenchyme. In vivo these cells undergo a mesenchymal to epithelial transformation during the early stages of nephron development. The P1 cell line is derived from adult mouse proximal tubule epithelium and the MDCK cell line is derived from an adult canine collecting duct epithelium. The epithelial characteristics of these cell lines make them
appropriate models for testing the function of ret, since in vivo, ret is expressed by the ureteric bud/epithelium.

Both the M5 and the P1 cells express GDNFR\(\alpha\) but do not express GDNF, as assessed by RT-PCR (see Chapter 3). It was not possible to assay the MDCK cells for expression of either ret or GDNFR\(\alpha\) by RT-PCR, as the canine sequences for these genes are unknown. It was assumed that the cells are probably negative for ret since they are derived from an adult kidney, which is known to be devoid of ret expression (see Chapter 3). Controls containing an empty vector were performed to assess any changes in the cellular phenotype due to the transfection and cloning processes.

MDCK cells have been shown to exhibit a highly motile and invasive phenotype under specific conditions, in vitro. One of the growth factors which elicits this effect is HGF (Montesano et al., 1991). Under the conditions of in vitro organ culture, HGF is known to effect the branching morphogenesis of the metanephros (Woolf et al., 1995), in a similar manner to that described for GDNF (see Chapter 4). MDCK cells are therefore, an ideal model for testing the ability of GDNF to illicit branching morphogenesis, via the activation of ret.

The cell lines were transfected with the two constructs and the vector alone. A number of neo\(^R\) cells were cloned by ring cloning (as described Chapter 2) and screened for expression of either ret or RET/PTC2 by RT-PCR. Equal numbers of the positively expressing cells were pooled, and assayed for a change in phenotype under various two and three dimensional culture conditions. The cells were also assessed for a change in their rate of proliferation, in monolayer.

### 5.2 Results

#### 5.2.1 Establishment of stably transfected M5, P1, and MDCK cell lines with mouse ret and human RET/PTC2

The full length mouse ret and human RET/PTC2 cDNAs were subcloned in to pCI-neo, as described in Chapter 2 (Figure 5.1). Transfected cells were selected for by their ability to grow in media supplemented with G418. After two weeks of growth in G418 supplemented media, a number of neo\(^R\) clones were isolated by ring cloning.

Genomic DNA was isolated from all clones and assessed for integration of the expression vector by PCR. It was assumed that all positive clones had integrated the plasmid as any episomal DNA would be lost during the passaging of the cells from the original transfected cell to the cell line. Cells transfected with the vector alone were negative for both ret and GDNF. The genomic copy of ret could not be amplified because the primers were
designed to cross introns, thus making the product too large. The RET/PTC2 primers were designed for the human sequence and did not amplify mouse ret.

Individual clones were assessed for expression of either ret or RET/PTC2 by RT-PCR, using the primers for ret and RET/PTC2 described in Chapter 2. Genomic DNA contamination could lead to false positive results, as it now contained a cDNA copy of ret. For this reason controls, containing no RT enzyme in the RT reaction, were performed to check for DNA contamination.

Figure 5.1 PCI-neo vector
(A-F) RT-PCR of transfected cell lines. Each sample has two lanes with the first having no RT in the RT reaction to control for genomic DNA contamination. The last lane on each gel was a negative control and contained ddH2O. (A-C) RT-PCR using primers designed for ret. (A) P1 cells transfected with ret. (B) M5 cells transfected with ret. (C) MDCK cells transfected with ret. (D-F) RT-PCR using primers designed for RET/PTC2. (D) P1 cells transfected RET/PTC2. (E) M5 cells transfected with RET/PTC2. (F) MDCK cells transfected with RET/PTC2.
The RT-PCR results demonstrated that there were 5 P1 RET/PTC2, 6 M5 RET/PTC2, 5 MDCK RET/PTC2, 6 P1 ret, 7 M5 ret, and 9 MDCK ret expressing cell lines. Equal numbers of each of these clones were pooled, with respect to each of the genes and cell lines. This facilitated screening for a change in phenotype and averaged out slight phenotypic differences between the clones. At least 5 control clones were also pooled for each cell line.

5.2.2 Influence of the ret and RET/PTC2 genes on cell morphology in vitro

5.2.2.1 Phase contrast microscopy

All the transfected clones largely resembled the parental and control cell lines in monolayer cultures at subconfluent, and saturation densities. The P1 and MDCK cells grew as typical epithelial cells, with discrete, regular cell borders. At saturation levels both the control and experimental MDCK cells formed domes, due to the polarisation of the cells. The M5 cells continued to express a mesenchymal cell morphology with irregular cell borders. All cell lines underwent contact dependent growth inhibition at saturation levels.

5.2.2.2 Morphology of cells grown in Matrigel™ and collagen type I gels

The cells were seeded into the gels at $1 \times 10^4$ cells ml$^{-1}$ gel. In Matrigel™, all cell types, with both experimental and control vectors, grew as small compact colonies with a single or multiple lumena (Figure 5.2). However, none of the cell lines exhibited any invasiveness into the Matrigel™. These cultures were visualised under phase contrast microscopy. The addition of 100 ng ml$^{-1}$ GDNF to these cultures did not affect their morphology over a two week period.

In collagen type I, the P1 and M5 cells had a similar morphology to that seen in the Matrigel™ cultures. Again, the addition of 100 ng ml$^{-1}$ GDNF did not affect this morphology over a two week period.

After the transfection and cloning of the MDCK cells, their ability to respond to HGF was assessed, as described in (Montesano et al., 1991). Briefly, the cells were cultured in collagen type I gels over a feeder layer of Ras/3T3 cells. Ras/3T3 cells secrete HGF which stimulates the cells to undergo invasive tubular morphogenesis. When grown within collagen type I gels in the absence of HGF, the cells grew into large spherical cysts consisting of many cells, as already described (McAteer et al., 1987; Warren and Nelson, 1987) (Figure 5.3, F). Following the addition of HGF, extensive branching tubule formation occurred over the course of several days, in agreement with a previous report (Montesano et al., 1991). Both control, experimental, and parental MDCK cell lines exhibited this response (data not shown).
Figure 5.2 MDCK/ret cells grown in Matrigel™

MDCK/ret cells grew as multicellular cysts in the presence (A and B) and absence (C and D) of 100 ng ml⁻¹ GDNF. All micrographs are to the same scale. Bar in A=100 μm.
Figure 5.3 MDCK/ret cells grown in collagen type I

MDCK/ret cells grew as multicellular cysts in the absence of GDNF (F). (A-E) In the presence of 100 ng ml⁻¹ GDNF, they grew broad sharp projections which invaded the gel. Magnification is the same in all micrographs. Bar in A=100 μm.

(G) MDCK/ret cells grown in the presence of ras3T3 conditioned medium.
Figure 5.3b MDCK/ret or MDCK/RET/PTC2 cells do not exhibit increased motility in the presence of GDNF in wounding or scatter assays.

(A-C) Scatter assay. (A) MDCK/ret cells grew as compact colonies. (B) MDCK/ret cells scattered in the presence of ras3T3 conditioned medium. (C) MDCK/ret cells did not scatter in the presence of 100 ngml⁻¹ GDNF. (D-F) Wounding assay. (D) In control cultures the MDCK/ret cells did not migrate into the wounds. (E) In the presence of ras3T3 conditioned medium the MDCK/ret cells migrated into the wound but not in the presence of 100 ngml⁻¹ GDNF (F).
The ability of the control and experimental MDCK cells to invade collagen type I gels in response to GDNF was investigated and found to be markedly different (Figure 5.2, A-E). The control MDCK and MDCK/ret cells grew as simple spherical cysts in collagen type I in the absence of HGF or GDNF. The addition of 100 ng ml\(^{-1}\) GDNF stimulated the MDCK/ret cells, but not the controls, to invade the collagen to a small, but none the less, notable amount. However, this was clearly not to the level of the response to HGF. MDCK/RET/PTC2 cells also exhibited this phenotype in the absence of GDNF. Within a few days of growth in the presence of GDNF MDCK/ret cells formed small cysts with irregular edges and displayed apparent invasion into the surrounding matrix, with broad short projections. After 2 weeks in culture these structures had not grown to the complex branching tubules observed in the HGF treated cells.

5.2.2.3 MDCK/ret or MDCK/RET/PTC2 cells do not exhibit an increased motility in the presence of GDNF, in 2 dimensional cultures

In the absence of any growth factors, MDCK cells form tight discrete colonies under subconfluent culture conditions. When seeded in the presence of ras/3T3 conditioned medium and grown for 24 hours, MDCK control and experimental clones dispersed into individual, highly motile cells. Since HGF and GDNF have somewhat similar effects on renal development in organ culture (Woolf et al., 1995; Towers et al., 1997), it was postulated that GDNF may have a similar effect on MDCK/ret cells as HGF. However, neither controls nor experimental MDCK cells displayed any motile phenotype in response to GDNF, the colonies exhibited tight discrete borders, as seen when grown without any exogenous growth factor (data not shown).

The effect of GDNF on MDCK/ret and MDCK/RET/PTC2 cell migration was further investigated using a wounding assay. In the presence of ras/3T3 conditioned all of the MDCK cell lines showed migration into the wound over a 24 hour period. However, in the presence of GDNF, both controls and experimental MDCK cells failed to undergo any level of migration (data not shown).

Neither P1 nor M5 cells have ever been reported to exhibit any motile behaviour. Due to lack of time these clones were not assayed for a response in cell motility to GDNF. It is, however, unlikely that GDNF would cause an increase in motility in these cells as it does not do so in MDCK cultures.

5.2.2.4 GDNF does not effect cell survival in non-permissive culture conditions

The M5 and P1 cell lines are derived from a mouse harbouring the H-2K\(^b\)-tsA58 gene, a \(\gamma\)-interferon-inducible, temperature-sensitive mutant of the SV-40 T antigen (Jat et al., 1991).
Under permissive conditions (33°C in the presence of 40 U ml⁻¹ recombinant murine γ-interferon) the cells are immortal but under the non-permissive conditions (39°C in the absence of γ-interferon) the cells cease to proliferate and die within a few days (Woolf et al., 1995). M5 and P1 experimental and control cells were seeded at 1 x 10⁵ cells per well, in 6-well plates at 39°C, without the addition of γ-interferon. At 24 hour intervals the cells were removed by trypsination and live cells were counted after they had excluded trypan blue. Both ret, in the presence and absence of 100 ng ml⁻¹ GDNF, and RET/PTC2 failed to rescue the cells from death, under non-permissive conditions. After three days in culture all of the cells were dead.

5.2.2.5 Epithelial cells suffer growth retardation in response to GDNF

The effect of GDNF on the proliferation rate of the control and experimental cells expressing ret, along with the basal proliferation rate of the cells expressing RET/PTC2 was assessed using ³H-thymidine incorporation to measure DNA synthesis. This protocol was optimised for both plating cell number and time of growth in culture. Figure 5.3 illustrates one optimisation for time.

Under basal conditions, the experimental MDCK cells, expressing ret or RET/PTC2, showed no significant difference in their proliferation rate to that of the control cells (Figure 5.4). The cells transfected with RET/PTC2 show a great variation between individual wells, as indicated by the large standard deviation. This error was not substantially decreased with a larger sample number (n=24). The addition of 100 ng ml⁻¹ GDNF to the MDCK cultures produced a highly significant drop in the amount of incorporated ³H-thymidine in the MDCK/ret cells, suggesting that the cells had undergone a significant decrease in their proliferation rates. There was no change in the proliferation rate of the MDCK control cells in the presence of GDNF.

The P1 cells also exhibited proliferative inhibition in response to GDNF (Figure 5.5). Under permissive conditions, in the absence of exogenous GDNF, both the P1/ret and the P1/RET/PTC2 clones incorporated less ³H-thymidine than the control cells, with the P1/RET/PTC2 cells incorporating four fold less thymidine than the controls and P1/ret incorporating two fold less ³H-thymidine than the control cells. With the addition of 100 ng ml⁻¹ GDNF, the P1/ret cells underwent further proliferative inhibition to almost undetectable levels, whereas there was no effect on the control cells.
M5 cells were seeded at a density of 4,000 cells per well of a 96 well plate. The cells were then left for 24, 48, or 72 hours before the addition of the $^3$H-Thymidine and a further incubation of 24 hours. It can be seen from the graph that after a total incubation period of 48 hours the cells have not yet reached their maximum proliferation rate. At 72 hours the cells are proliferating maximally. At 96 hours the cells are starting to undergo contact inhibition, indicated by a drop in the rate of proliferation.

Error bars shown are standard error.
Figure 5.5 Graph showing the proliferation of MDCK cells

(*) Experiment 1; (**) Experiment 2. Experiments 1 and 2 are not directly comparable as they used different $^3$H-thymidine aliquots.

Error bars shown are standard error.
Figure 5.6 Graph showing the proliferation of P1 cells

Error bars shown are standard error.
Figure 5.7 Graph showing the proliferation of M5 cells

Error bars shown are standard error.
The M5/RET/PTC2 cells were also severely affected, incorporating very low levels of thymidine compared to the control (Figure 5.6). However, the M5/ret cells exhibited no significant change in $^3$H-thymidine incorporation in comparison to the control cells in both the basal and experimental conditions.

5.3 Discussion

GDNF is involved in many cellular phenomena, however, its exact role within these biological processes is yet to be determined. Of particular interest, is the role of GDNF in mammalian metanephric development, as discussed in Chapter 4, and previous work (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996; Vega et al., 1996). For these reasons a study into the effect of ret activation by GDNF in several kidney cell lines was undertaken.

The proliferation assays of the ret transfected cells illustrate that GDNF activation of ret causes a highly significant drop in the measurable level of DNA synthesis suggesting a decrease in the rate of proliferation. The transfection of the PI cells with ret caused a two fold drop in cellular proliferation. The addition of exogenous GDNF to these cells caused an almost total cessation in cellular proliferation. The PI cells are known to express endogenous GDNF, as discussed in Chapter 3. This endogenous GDNF probably acts in an autocrine manner to effect the proliferation rate of the PI/ret cells. It is important to note that the growth inhibition caused by GDNF appears to be dose dependent as the addition of exogenous GDNF further inhibits cellular proliferation. Inhibition of proliferation is also seen in the MDCK/ret cells, however, it can be assumed that these cells do not release endogenous GDNF, as only in the presence of exogenous GDNF is any growth inhibition detected.

GDNF is a distant member of the TGFβ super family (Lin et al., 1994). TGFβ is a known inhibitor of cellular proliferation of a variety of cell types including normal mammary epithelial cells where it also causes differentiation (Miettenen et al., 1994). Markers of differentiation were not examined in this study but one could postulate that the growth inhibition is due to a more differentiated phenotype, and this could be investigated.

The MDCK cell line has been used as a model cell line to investigate a variety of responses to biological factors in vitro, including cell scattering and motility (Stoker et al., 1987), matrix invasion (Weidner et al., 1990), and tubulogenesis (Montesano et al., 1991). Previous studies have shown that the organisational behaviour of MDCK cells cultured within collagen gels can be experimentally altered. Low level expression of the viral tyrosine kinase pp60$^{v-src}$ induces the formation of elongated cell processes, or 'spikes', projecting from the
basal surface of the cysts (Warren and Nelson, 1987), in a similar fashion to the phenotype of the ret/MDCK cells described here. Elevated expression of the cellular tyrosine kinase pp60c-src results in the formation of irregularly shaped cysts with a distorted architecture, although there is no tubule formation (Warren et al., 1988). From these findings it appears that MDCK cells have the potential to express at least two distinct morphogenetic programs, the formation of cysts, or branching tubules, with an array of invasiveness. The particular pathway followed is determined by environmental signals, as discussed in (Montesano et al., 1991). This three dimensional organisation is paralleled in monolayer by the ability of MDCK cells to grow as tight epithelial islands, or scattered fibroblastic individual cells (Stoker et al., 1987). MDCK cells have been utilised in this study, to investigate the cellular effect of ret activation, by GDNF, in mammalian renal epithelial cells.

The activation of ret in MDCK cells causes a change in the morphogenetic program from the formation of cysts to that of invasive cell processes. The phenotype exhibited by these ret positive cells in response to GDNF is not as dramatic as that seen in response to HGF. This limited phenotype may be due to the inhibition of proliferation caused by GDNF. The change in cell morphology, in collagen gel, suggests that the function of GDNF is to initiate branching morphogenesis, with the initial stages being independent of cell division, as described for the submandibular salivary gland cleft formation (Spooner et al., 1989). However, without the addition of external growth factors the full phenotype of tubulogenesis is not observed.

The finding that GDNF causes a dose dependent inhibition of proliferation appears to contradict the findings described in Chapter 4, along with other work from our laboratory (Towers et al., 1997). Ureteric bud cells grown as primary cultures in monolayer exhibit increased cell survival in the presence of GDNF. They also exhibit increased proliferation rates as compared to cultures grown in the absence of GDNF, as assessed by BrdU incorporation (Towers et al., 1997). The contradiction between these findings and the findings described here, may be explained as follows. It was noted, although not quantified, that the cells transfected with ret or RET/PTC2 did not adhere to the plastic quite as well as the control cells, with more cells seen floating in the medium. This suggests that the activation of ret may be increasing the motility of the cells by decreasing their adhesion. If this is truly the case, the measured uptake of 3H-thymidine would not represent a true proliferation value, as many of the cells would not be counted, as they were not adhered to the wells. This possibility could be investigated further by quantifying the population of floating cells, with a cyto-spin. The collected cells could be stained and counted. An alternative theory is that the effect of GDNF on the ureteric bud cell cultures was to increase the survival of these cells, with an increase in proliferation being a secondary effect. Future work will clarify these points.
5.4 Further work

In this study there has been no direct measurement of ret protein levels, or the level of phosphorylation of ret in response to GDNF. Very low levels of ret protein may explain the "spike" phenotype, as compared to the tubulogenesis seen in response to HGF. These questions could be answered by quantitative analysis of the levels of expression and translation of ret and RET/PTC2 using Northern and Western blots. Immunohistochemistry with anti-ret antibodies would confirm the correct cellular localisation of the proteins.

The inhibition of growth in response to GDNF may indicate a more differentiated phenotype, as seen in the response to TGFβ by mammary epithelial cells (Miettenen et al., 1994). There are many markers of induction and differentiation in the kidney, as reviewed in (Davies, 1993). Immunohistochemistry could be used to screen for the presence of some of these protein markers to confirm a more differentiated phenotype.

Work described previously in chapter 4, suggested that GDNF works in a dose dependent manner. It has been demonstrated here that the addition of exogenous GDNF, to the proposed endogenous GDNF, causes an increase in growth inhibition. However, a direct measurement of secreted bioactive GDNF has not been made. It has been reported that over-expression of the receptor tyrosine kinase ron, causes receptor dimerisation due to the high numbers of receptor proteins present (Gaudino et al., 1994). The CMV promoter of the pCI-neo construct should produce a high level of expression in mouse cells. If this is the case, then a degree of receptor dimerisation due to the presence of a high number of receptor molecules may occur. This concept may explain the growth inhibition in the P1/ret cells grown in the absence of exogenous GNDF. This hypothesis could be tested by the addition of blocking antibodies to P1/ret cells. Any ligand related growth inhibition, of the P1/ret cells would then be blocked under these conditions.
6. Morphological and partial immunocytochemical characterisation of renal mesenchymal spheroids

6.1 Introduction

Mammalian renal development relies upon mutual induction between the ureteric bud epithelial cells and the mesenchymal cells of the metanephric blastema. Some of the metanephric mesenchymal progenitor cells function as stem cells. They are a self renewing population which give rise to the diverse epithelial phenotypes of the adult kidney, possibly including the collecting duct system (Qiao et al., 1995). Murine organ culture techniques and transgenic technology have established some of the molecules involved in the processes of mesenchyme condensation and differentiation into polarised epithelial cells. However, much of the molecular basis remains to be established, reviewed in (Davies, 1993; Bard et al., 1994).

The undifferentiated mesenchymal cell population is characterised by active proliferation in vivo (Magauer and Ekblom, 1991). This must be stringently regulated to maintain a proper balance between the self-renewing stem cell population and its differentiated progeny. A cell culture system of proliferating undifferentiated renal mesenchymal cells offers the opportunity to study the molecular basis of mesenchymal proliferation and mesenchymal to epithelial transition. The mesenchymal cell line M5, used in this study, is thought to represent an extremely primitive mesenchymal cell. It expresses the mesenchymal cell marker vimentin, which is replaced by cytokeratin as the cell differentiates into epithelia, in vivo. M5 cells also express the matrix receptor syndecan but not E-cadherin, as predicted for undifferentiated mesenchymal cells of the mouse metanephros.

In monolayer cultures, a widely used in vitro system for the study of cell physiology, the in vivo arrangement of cells is not seen and although some cell-cell and cell-substratum contacts are made, the precise intercellular relationships present in situ are lost. For this reason a three dimensional culture system appears to be more representative of the in vivo situation. A number of studies (Moscona, 1961; Sutherland and Durand, 1976) have shown that cells require a proper three dimensional cyto-architecture, as found in vivo, for optimal functioning. Gover et al. demonstrated that the expression of specific visceral endoderm functions by F9 embryocarcinoma cells was dependent upon the formation of a proper basement membrane. They demonstrated that not only laminin and fibronectin, produced by the cells, were essential
for their differentiation but also that the insoluble proteins had to be deposited in a very specific pattern, a pattern not seen in two dimensional culture (Grover et al., 1997).

As well as the loss of the in vivo cyto-architecture, cells in monolayer form limited degree of cell-cell contacts and not the high level of homologous and heterologous cell-cell contacts found in vivo. It is well acknowledged that a variety of positive, as well as negative, signals regulating cell growth and differentiation can be provided by intercellular contacts (Glaser, 1982). Studies on early kidney development indicate that condensation and polarisation of the mesenchyme requires cell-cell and cell-substratum adhesion. During condensation of the mesenchyme many cell-cell contacts are made. Conversion into polarised epithelia is later mediated by cell-substratum interactions with the ECM (Sorokin et al., 1990).

Epithelial cells are polarised into discrete apical, lateral, and basal domains. Specialised structures such as microvilli and cilia extend from the apical pole; the Golgi complex is positioned in the apical cytoplasm. Tight junctions, and desmosomes are found on lateral borders that also contain cell adhesion molecules, such as E-cadherin, that mediate interactions with neighbouring cells. Basal plasma membranes contain integrin receptors (Rouslahti, 1991) that interact with the ECM in the underlying basement membrane. Some integrins may also mediate cell-cell adhesion (Larja et al., 1990; Carter et al., 1990).

A number of studies have shown that E-cadherin plays a role in cell-cell contacts made during polarisation (Nelson and Veshnock, 1987; McNeil et al., 1990). It is demonstrated here that E-cadherin is upregulated during spheroid formation.

### 6.2 Results

The culture system used here has been described in detail in the methods section of Chapter 2. In summary, M5 cells were prevented from adhering to the plastic of the culture dish by a layer of agar to which the cells show little adhesiveness. Under these conditions the cells underwent dramatic aggregation and reorganisation.

#### 6.2.1 Spheroid formation and development

The embryonic mesenchymal cell line M5, used in this study, grows as an adherent monolayer with irregular outlines under normal permissive culture conditions (Figure 6.1, A). When plated onto agar coated dishes the cells clustered into spherical aggregates after 24 hours, with a maximum size of 150-175 μm (Figure 6.1, B).

During the first few hours of culture the cells were loosely attached to each other and could easily be separated by gentle pipetting. However, after 24 hours in culture these weak bonds had been replaced by tighter bonds so that the cells could no longer be released by
pipetting. The spheroids now appeared as more compact structures in which individual cells could hardly be distinguished under phase-contrast microscopy. The compaction process appeared to be complete by 48 hours, after which time there was little change in general morphology. There was, however, a slow and limited increase in diameter. Although no attempt was made to directly assess whether cell division was responsible for the increase in size, many mitotic bodies were seen during the histological analysis. The total numbers of free floating cells appeared to decrease over time, so that after 2 weeks in culture few single cells could be seen. This suggests that either these cells died or that a continuous recruitment of single cells into the spheroids was occurring.

6.2.2 Histological analysis

All the histological analysis was carried out on spheroids cultured for 48 hours, except for those cultured for two weeks in order to investigate cell survival in long term cultures. The histology of the spheroids was examined by H and E staining of fresh and fixed cryostat sections (Figure 6.1, C), confocal microscopy of propidium iodide (PI) stained spheroids (Figure 6.1, D), and by transmission electron microscopy of ultra thin sections (Figure 6.2).

At the cellular level all cells appeared to have a normal morphology, with distinct cell borders, containing one nucleus with one or two dark nucleoli. Interestingly, within the spheroid there was often the appearance lumena, these were either single or multiple. Ultrastructural analysis by transmission electron microscopy failed to detect these lumena, although, this was probably due to the processing procedures. However, it was possible to see areas were the lumena may have been. Figure 6.2 B shows such a spheroid with a central 'collapsed' lumen. The cells surrounding the lumena show typical features of polarised epithelia. There were basely located nuclei, and villi present on the apical surface. Tight junctions can also be seen, located on the lateral sides of these polarised cells. Figure 6.1 D-F shows a three dimensional reconstruction of a lumen, as seen with the confocal microscope.

6.2.3 Cell survival

While most of the histological observations reported above were performed at 48 hours, observations made at later times revealed the same apparent integrity at the structural level for at least 2 weeks. A direct assessment of cell survival in spheroids was performed by electron microscopy where no obvious areas of necrosis or apoptosis could be seen.
Figure 6.1 Spheroid formation

(A) M5 cells in monolayer stained for vimentin. (B) Light micrograph of whole spheroid in culture. (C) H and E stained fixed cryostat section of and organoid. Note the presence of lumena. (D-F) Confocal images of PI stained organoid with a central lumen (L). (E and F) Verticle and horizontal reconstructions of serial optical sections through the lumen. Bar in A=20 μm. (B and C) are the same magnification. Bar in B=100 μm. (D, E, and F) are the same magnification. Bar in D=20 μm.
Figure 6.2 Electron micrograph of spheriods
Figure 6.2 Electron micrograph of spheriods

(A) Figure showing the normal cellular morphology of the cells within the spheroid. (N) nucleus; arrows point to intercellular junction. (B) This micrograph demonstrates a collapsed lumen (L). Villi (V) are located on the apical side of the cells with the nuclei (N) being located basolaterally. Bar in A=2 μm. Bar in B=4 μm.
6.2.4 Expression of E-cadherin is upregulated in spheroids

E-cadherin is a marker of mesenchymal induction, its expression was examined in monolayer and spheroid cultures. E-cadherin could not be detected in monolayer cultures by immunocytochemistry, but by confocal analysis E-cadherin was detected in spheroids (Figure 6.3). The E-cadherin protein was not properly localised to the cell membranes, as is seen in
6.3 Discussion

The biochemical basis for cell aggregation and reorganisation in three dimensional structures has been extensively studied (Glaser, 1982; Harrison and Chesherton, 1980; Garrod and Nicol, 1981). The aggregation process described here appears to be composed of two phases. The initial phase probably results from direct chemical or physicochemical interactions among specific cell surface determinants already present on the cells. The second phase is characterised by the compaction of the spheroids and probably results from the formation of specialised junctions, such as tight junctions, between the cells. The fact that the cells are originally only loosely attached suggests that such junctions are not present in the early stages of aggregation, as the lack of E-cadherin in monolayer cultures suggests. These same junctions are seen in condensing mesenchyme, in vivo, suggesting that the three dimensional structure of the spheroids permits the cells to differentiate in a similar manner to that seen in vivo.

From the dramatic reorganisation of the cells in the spheroid culture it seems likely that basolateral plasma membranes may start to express markers of a more differentiated phenotype. It is well documented that E-cadherin has a role in epithelial polarisation (McNeil et al., 1990) and is expressed by the metanephric mesenchymal cells as they differentiate into nephrons.

In this model the accumulation and specific deposition of ECM materials is likely to be involved in the expression of a more differentiated phenotype. The precise geometrical deposition of ECM molecules relative to the cells is important in their differentiation (Grover et al., 1997). As discussed in the introduction the three dimension culture allows a higher level of homologous and heterologous cell-cell contacts mimicking the in vivo state more closely.

Apart from its use as a new in vitro model for the investigation of problems specific to the kidney, this system provides a unique opportunity for studying the role(s) of factors involved in the establishment and maintenance of three dimensional structures. The system
used here is ideal for identifying specific inducers, as controlled media can be used. The model of a clonal embryonic mesenchyme cell line is ideal for elucidating factors involved in the condensation of metanephric mesenchyme, followed by mesenchymal to epithelial conversion.

6.4 Further work

It has been postulated above that the condensation of the spheroids into compact structures may involve cadherins, calcium-dependent adhesion molecules (Takeichi, 1991). It would therefore, be of interest to investigate the effect of growing these cells in low calcium media to investigate the role of cadherins in the formation of spheroids.

The cells described in the previous chapter harbour an expression vector containing ret. Ret is known to contain a sequence of 110 amino acids that is homologous to a domain found in all vertebrate cadherins. This motif is thought to be a putative calcium binding site that is important for the homophilic binding of cadherins (Iwamoto et al., 1993). Although there has been no evidence that ret is involved in cell to cell adhesion it would be interesting to investigate how these cells behave in the aggregation assay described here, especially when considering that the M5 cells are known to express GDNF and GDNFRα. If ret is involved in the branching morphogenesis of cells one could hypothesis that ret may not increase the aggregation exhibited by the M5 cells, but may affect the overall morphology of the spheroids.
7. Final Discussion

Many organs within the metazoan body develop by a process of branching morphogenesis. This process is guided by inductive interactions between the epithelial and mesenchymal lineages. For many years the molecules involved in this inductive interaction remained an enigma. However, advancing techniques such as transgenic technology, have started to shed light onto the signalling pathways involved. One of these pathways is the ret-GDNF signalling pathway (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Moore et al., 1996).

Ret is a receptor tyrosine kinase which is expressed by the epithelial components of both the metanephros and lung, organs which are known to develop by branching morphogenesis. Its ligand, GDNF, was initially isolated as a neurotrophic factor and is expressed by the mesenchyme of many organs that develop by branching morphogenesis (Lin et al., 1994; Hellmich et al., 1996). The interaction between GDNF and ret is mediated by a further molecule, GDNFRα. GDNFRα is a glycosylphosphatidylinositol-linked cell surface receptor and is expressed by both epithelial and mesenchymal cells (Jing et al., 1996; Treanor et al., 1996).

The effect of exogenous GDNF on organ cultures of metanephroi and lung primordia is highly specific. In vitro and in vivo, only the development of the metanephros is affected by manipulations of the pathway in either null mutant mice or organ culture. Mice harbouring null mutations in either the ret or GDNF gene exhibit severe renal malformations with no abnormality being reported in the lung. In GDNF treated organ cultures the metanephros exhibited accelerated development with a significant increase in the numbers of branch tips and a corresponding increase in nephron formation, with blocking antibodies having the converse effect. Cultures of lung primordia under the equivalent conditions develop normally. Interestingly, the addition of exogenous GDNF also caused the production of ectopic buds.

From the results of this project several conclusions can be drawn. It has been demonstrated that the Wolffian duct is already expressing ret when it comes into contact with the metanephric blastema and it is the local concentration of GDNF, captured and presented to ret by GDNFRα, that induces and directs the evagination of the ureteric bud. As development progresses GDNF is prevented from having any further effect on the Wolffian duct due to its sequestration in the nephrogenic zone by GDNFRα.

Grobstein originally hypothesised that there are mesenchymal specific and mesenchymal common factors. It appears that the ret-GDNF signalling system is a common pathway to both the metanephros and the lung. However, the organotypic expression pattern of
the individual components of the pathway are reflected in the organotypic developmental role of the pathway within these organs. From these results Grobsteins original hypothesis can be seen to be somewhat simplistic and it is the specific spatial and temporal expression of the individual components of any pathway that determine its function within the development of a particular organ.

GDNFRα is a necessary component of the interaction between ret and GDNF (Jing et al., 1996; Treanor et al., 1996). Lin et al. (1994) and Trupp et al. (1996) have reported different binding affinities between GDNF and GDNFRα. These findings suggest that there may be different isoforms of GDNFRα which act to regulate the concentration of GDNF necessary to elicit the activation of ret. The differential expression of such isoforms in the lung and metanephros may explain the lack of effect of GDNF in lung cultures. The expression of a high affinity GDNFRα isoform in the lung would indicate that very low levels of GDNF would be sufficient to activate ret. Hence, the addition of exogenous GDNF would have no effect. In contrast the expression of a low affinity receptor in the metanephros would ensure that the evagination of the ureteric bud would occur at the position closest to the metanephric blastema and thus ensuring growth of the ureteric bud into the blastema.

Results described in Chapter 5 demonstrate that GDNF induced activation of ret causes a decrease in thymidine incorporation. This data appears to contradict the findings from both the organ culture experiments, where GDNF causes accelerated development, and the invasive phenotype of the MDCK/ret cells grown in collagen type I gels. It has previously been reported that the growth and branching of the ureteric bud is under the control of separate pathways (Davies et al., 1995). It is therefore, possible that GDNF affects the process of branching only, with the level of proliferation being controlled and sustained only in the presence of another factor, such as HGF (Woolf et al., 1995). In the case of the organ culture GDNF is the rate limiting factor in metanephric development so the addition of exogenous GDNF releases the organs to develop at an increased rate.

Finally, a three dimensional culture system has been described. This will be utilised in future work to elucidate further the exact role of signalling pathways during organogenesis.
Appendix 1

Expression in the ectodermal placodes and their neurogenic derivatives

Neural crest cells migrating from rhombomere 4 into the second branchial arch have previously been reported to express ret, with the second branchial arch and more posterior arches also showing positive expression (Pachnis et al., 1993). Figure A1 illustrates preliminary expression data for GDNFRα. GDNF was expressed in a well defined domain in structures which are presumed to be the first and second cleft endoderm.

The otic placode arises adjacent to the mid-myecephalon (rhombomeres 5 and 6) shortly after the initiation of somite formation. After regionalised specialisation it gives rise to the vestibular and auditory neurons associated with the VIIIth ganglia. At E10, the dorsal epithelia of the otic placode was positive for GDNFRα (Figure A2), with the ventral epithelia expressing GDNF (Figure A1, D). However, expression of ret was not detected in either the epithelia or associated mesenchyme.

The expression of GDNF in the epithelia of the branchial placodes at E10 suggests that it may represent a very early marker of placodal specification, or even a marker of undifferentiated placodal cells. Studies using younger embryos would be necessary to confirm this hypothesis.

Figure A1 Expression of GDNF in developing cranial structures

(P) pharynx; (BA) branchial arches; (OV) otic ventricle. Arrows point to epithelial expression. Bar in A=40 μm, bar in B and C=80 μm, bar in D=20 μm.

Figure A2 Expression of GDNFRα and ret in developing cranial structures

(A and B) GDNFRα, (C) ret. (OV) otic vesicle, (BA) branchial arches. Bar in A and B=80μm, bar in C=20 μm.
Figure A1 Expression of GDNF in developing cranial structures
Figure A2 Expression of GDNFRα and ret in developing cranial structures
Appendix 2: Solutions

A2.1 General solutions

0.5 M EDTA
186.1 g EDTA
Dissolved 800 ml ddH₂O
The pH was adjusted to 8.0 with NaOH, the volume made up to 1 L and autoclaved.

4 % Paraformaldehyde-PFA
2 g of PFA in 50 ml ddH₂O, the solution was warmed in a 65°C waterbath in order to allow the PFA to dissolve.

1 X Phosphate buffered saline-PBS
10 pellets of dry PBS per Litre
1 L ddH₂O
The solution was then autoclaved.

5 M NaCl
292.2 g NaCl
Dissolved is 800 ml of ddH₂O, the volume made up to 1 L and autoclaved.

20 X SSC
350.5 g NaCl (3 M)
176.5 g Sodium citrate (0.3 M)
Dissolved in 1 L ddH₂O, the volume made up to 2 L and autoclaved.

10 % SDS-Sodium dodecyl sulphate
50 g SDS
450 ml of ddH₂O was added and the solution warmed gently in a 65°C waterbath to dissolve. Once cool the volume was make up to 500 ml.

TE buffer
0.6 g Tris (10 mM)
0.186 g EDTA (1 mM)
Dissolved in 250 ml ddH2O and pH adjusted to 8.0 with HCl. The volume was made up to 500 ml and autoclaved.

1 M Tris
121.1 g Tris
Dissolved in 800 ml of ddH2O, the pH was adjusted to the desired value by adding HCl. The volume was made up to 1 L and autoclaved.

10 X Tris buffered saline-TBS
80 g NaCl
2 g KCl
3 g Tris
Dissolve in 800 ml ddH2O, adjust pH to 7.4 with HCl. The volume was made up to 1 L and autoclaved.

A2.2 Solutions for agarose gel electrophoresis
50 X TAE
242 g Tris
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8)
Volume made up to 1 L with ddH2O.

Loading buffer
0.25 % Bromophenol blue
10 % glycerol

A2.3 Solutions for preparation of plasmid DNA
LB-Lennnox broth
20 g LB
Dissolved in 1 L ddH2O and autoclaved.
LB agar
2 g LB
1.5 g Select agar
Dissolved in 100 ml ddH$_2$O and autoclaved. The flask was allowed to cool. Once cool 50 μg ml$^{-1}$ ampicillin and 50 μg ml$^{-1}$ methicillin was added. The agar was then poured into petri dishes using aseptic technique. Plates were left to dry before storing inverted at 4°C.
Solution I
0.3 g Tris (25 mM)
0.37 g EDTA (10 mM)
0.9 g Glucose (50 mM)
Dissolved in 100 ml ddH₂O, autoclaved and stored at 4°C.

Solution II
0.1 ml 10 N NaOH (0.2 N)
0.5 ml 10 % SDS (1 %)
Volume was made up to 5 ml with ddH₂O. This must be prepared fresh at the time of use.

Solution III
60 ml 5 M Potassium acetate
11.5 ml Glacial acetic acid
28.5 ml ddH₂O
Stored at 4°C

A2.4 In situ hybridisation solutions

PBT
10 tablets of PBS were dissolved in 1 L of ddH₂O. 500 μl of DEPC was then added, the solution was left in the fume hood for a few hours before autoclaving to remove the DEPC. After autoclaving the solution was left to cool and then 1 ml of Tween-20 was added.

Prehybridisation Mix
50 % formamide
5 X SSC (pH 4.5 with citric acid)
50 μg ml⁻¹ yeast RNA
1 % SDS
50 μg ml⁻¹ heparin
These were made up with ddH₂O to the required volume.
TBST (10 X) 100 ml
8 g NaCl
0.2 g KCl
25 ml 1 M Tris-HCl pH 7.5
The NaCl and KCl were dissolved in 50 ml ddH₂O. The Tris was then added and the volume made up to 100 ml before autoclaving.

TBST Working Solution 50 ml
5 ml 10 X TBST
0.5 ml Tween-20
0.024 g Levamisole
44.5 ml H₂O
This solution was made up fresh on the day of use.

NTMT working solution 50 ml
1.2 ml 5 M NaCl
2.5 ml 2 M Tris-HCl pH 9.5
1.25 ml 2 M MgCl₂
0.5 ml Tween-20
0.024 g Levamisole
This solution was made up fresh on the day of use.

TE-DEPC
10 mM Tris pH 8
1 mM EDTA
The Tris and EDTA were dissolved in DEPC treated ddH₂O before autoclaving.

Goat serum
The serum was heat treated at 56°C for 30 minutes before use.

Proteinase K solution
20 μg ml⁻¹ Proteinase K (Sigma) from 10 mg ml⁻¹ stock
50 mM Tris from 1 M stock
5 mM EDTA from 0.5 M stock

**Embedding mixture for the vibrotome**

4.5 g Gelatine 300 Bloom

270 g Albumin Grade II (sigma)

180 g Sucrose

The gelatine was heated in 100 ml PBS with stirring until dissolved. It was then made up to 800 ml with PBS. The albumin was added and left stirring for several hours to dissolve. The sucrose was added and stirring continued until dissolved. It was aliquoted and stored at -20°C.

**A2.5 Western blotting solutions**

**8 % Running SDS-polyacrylamide gel**

5.3 ml ddH$_2$O

2 ml 30 % Acrylamide mix

2.5 ml 1.5 M Tris (pH 8.8)

0.1 ml 10 % SDS

0.1 ml 10 % Ammonium persulfate

0.008 ml TEMED

**5 % Stacking SDS-polyacrylamide gel**

3.4 ml ddH$_2$O

0.83 ml 30 % Acrylamide mix

0.63 ml 1 M Tris (pH 6.8)

0.05 ml 10 % SDS

0.05 ml 10 % Ammonium persulfate

0.005 ml TEMED

**5 X Running buffer**

9 g Tris

43.2 g Glycine

30 ml 10 % SDS
Dissolve Tris and Glycine in 500 ml ddH$_2$O before adding the SDS.

Volume made up to 600 ml
Bibliography


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