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CHARACTERIZATION OF THE *MET*/HGF/SF RECEPTOR TYROSINE KINASE IN CELL DIFFERENTIATION AND DURING MOUSE DEVELOPMENT

by

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A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

The *met* proto-oncogene receptor tyrosine kinase has been identified as the receptor for hepatocyte growth factor (HGF)/scatter factor (SF). HGF/SF is a multifunctional cytokine that is expressed predominantly by mesenchymal cells and stimulates mitogenesis, motogenesis, morphogenesis of a broad spectrum of epithelial and endothelial cells in culture. To elucidate the normal physiological role for the *met* receptor (R) in the adult mouse, I first determined the cell-type specific expression of the *met* R in adult mouse tissues by *in situ* hybridization. I have demonstrated that the *met* R is generally expressed in epithelial cells of many tissues, as well as in hepatocytes. This data supports a possible role for HGF/SF and the *met* R in epithelial cell renewal and tissue homeostasis. I also demonstrated that *met* R mRNA and protein, as well as its ligand were expressed at a low level in undifferentiated P19 cells. Expression was increased as those cells were induced to differentiate into neuroectodermal derivatives following treatment with retinoic acid (RA) and into mesodermal derivatives following treatment with dimethyl sulphoxide (DMSO). In cultures treated with RA and cytosine arabinoside, both *met* R mRNA and protein were localized specifically to non-dividing neuronal cells. Expression of the *met* R in undifferentiated P19 cells and differentiated derivatives supports a role of *met* R in the control of cell differentiation, proliferation during embryogenesis. I therefore examined the role played by *met* R during mouse embryogenesis using whole mount *in situ* hybridization. I have localized *met* R expression to the ventrolateral and dorsal-medial portions of the somite dermamyotome, in epithelial cells of the primitive gut and nephrogenic cords (the primitive urinary tract), in neuroepithelium of mouse embryos of 9 and 10 day gestation. Together, these data support a role for the *met* R in cell migration, proliferation and tubulogenesis during embryogenesis.

Résumé

Le proto-oncogène *met* encode un récepteur tyrosine-kinase qui a été identifié comme étant le récepteur du facteur de croissance hépatocytaire/facteur de dispersion (HGF/SF). Le HGF/SF est une cytokine multifonctionnelle principalement exprimée par les cellules mésenchymateuses. Sa liaison au récepteur *met* stimule la mitogénèse, la motogénèse et la morphogénèse chez plusieurs types de cellules épithéliales et endothéliales en culture. Afin d'élucider le rôle physiologique normal du récepteur *met* chez la souris adulte, j'ai tout d'abord déterminé la spécificité cellulaire de l'expression de *met* dans les tissus de souris adultes par hybridation *in situ*. J'ai montré que le récepteur *met* est généralement exprimé dans les cellules épithéliales de plusieurs tissus ainsi que dans les hépatocytes. Ces résultats suggèrent un rôle possible du facteur HGF/SF et du récepteur *met* au niveau du renouvellement cellulaire et de l'homéostasie tissulaire. J'ai également montré que les ARNm et les protéines *met*, ainsi que ceux de son ligand, sont faiblement exprimés dans les cellules P19 non-différenciées. Leur expression est augmentée lorsque ces cellules sont induites à se différencier en cellules du neuroectoderme suite à un traitement par l'acide rétinoïque (AR) et en cellules du mésoderme suite à un traitement avec le diméthyl sulphoxide (DMSO). Dans des cultures traitées avec l'AR et la cytosine arabinoside, les ARNm et les protéines *met* sont spécifiquement localisés dans les cellules neuronales quiescentes. L'expression du récepteur *met* dans les cellules P19 non-différenciées ainsi que chez les dérivés différenciés suggèrent un rôle pour *met* dans le contrôle de la différenciation cellulaire et de la prolifération durant l'embryogénèse. J'ai donc examiné le rôle joué par *met* lors de l'embryogénèse chez la souris par hybridation *in situ* sur l'embryon complet ("whole mount"). J'ai détecté l'expression du

récepteur *met* au niveau des portions ventrolatérales et dorsales médianes du dermamyotome des somites, dans les cellules épithéliales de l'intestin primitif et des cordes néphrogéniques (appareil urinaire primitif), ainsi que dans le neuroépithélium d'embryons de souris de 9 et 10 jours de gestation. Dans l'ensemble, ces résultats suggèrent un rôle pour le récepteur *met* dans la migration cellulaire, la prolifération et la tubulogénèse durant l'embryogénèse.

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Preface

The Guidelines Concerning Thesis Preparation Issued By The Faculty Of Graduate Studies And Research At McGill University reads as follows:

> "The candidate has the option, subject to the approval of their department, of including as part of the thesis, copies of the text of a papers(s) submitted for publication, or clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
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> The thesis must conform tc all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final conclusion and/or summary.
> Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g.. in the appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

I have chosen to write my thesis according to the above quoted option with one paper published, one submitted for publication and one to be submitted for publication, as well as some unpublished results. The thesis is organized into six chapters . Chapter I is a general introduction and literature review with references. Chapters II-IV contain manuscripts, each with its own abstract, introduction, methods, results and references. Chapter V is a general discussion of all the results with references and Chapter VI contains my claims to original research.

Publications arising from work of the thesis

1. **Yang, X.-M.** and Park, M. (1993). Expression of the *met*/hepatocyte growth factor/scatter factor receptor and its ligand during differentiation of murine P19 embryonal carcinoma cells. Devel. Biol. 157, 308-320.

2. **Yang, X.-M.** and Park, M. (1994). Expression of the hepatocyte growth factor/scatter factor receptor tyrosine kinase is localized to epithelia in the adult mouse. Oncogene (submitted).

3. **Yang, X.-M.** and Park, M. (1994). Characterization of the *met*/hepatocyte growth factor receptor in early mouse development by whole mount *in situ* hybridization. Development (to be submitted).

Publications arising from this work are not included in this thesis.

1. Santos, O.F., Barros, E.J., **Yang, X.-M.**, Matsumoto, K., Nakamura, T., Park, M. and Nigam, S.K. (1994). Involvement of hepatocyte growth factor in kidney development. Devel. Biol. 163(2): 525-529.

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Chapter I Literature Review.

1. Receptor tyrosine kinases (RTK)s and growth factors

1.1. Introduction.

Polypeptide growth factors and their receptors are thought to govern a variety of physiological processes such as cell growth, differentiation, embryogenesis, hematopoiesis and wound repair (Cross and Dexter, 1991; Adamson, 1993). One of the major classes of growth factor receptors are receptor tyrosine kinases (RTK) which are characterized by an extracellular ligand binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine specific protein kinase domain (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). Once bound to its respective ligand, the receptor initiates a series of signaling events through interaction with, and in some cases tyrosine phosphorylation of, specific downstream substrates, which in turn transmit the signal to the cell nucleus (Schlessinger and Ullrich, 1992). Many genes in this family were initially identified as activated oncogenes which, through constitutive tyrosine kinase activity, cause deregulation of their normal signaling pathways, resulting in uncontrolled cell growth and transformation (Cantley et al., 1991; Rodrigues and Park, 1994).

1.2. Classification of RTKs and growth factors.

On the basis of sequence similarity in the kinase domain and structural characteristics, it is possible to classify the growth factor RTKs into nine subclasses (Fig. 1) (Fantl et al., 1993).

Subclass I which includes the epidermal growth factor receptor (EGFR), neu and c-erbB receptors, are monomeric receptors, characterized by the

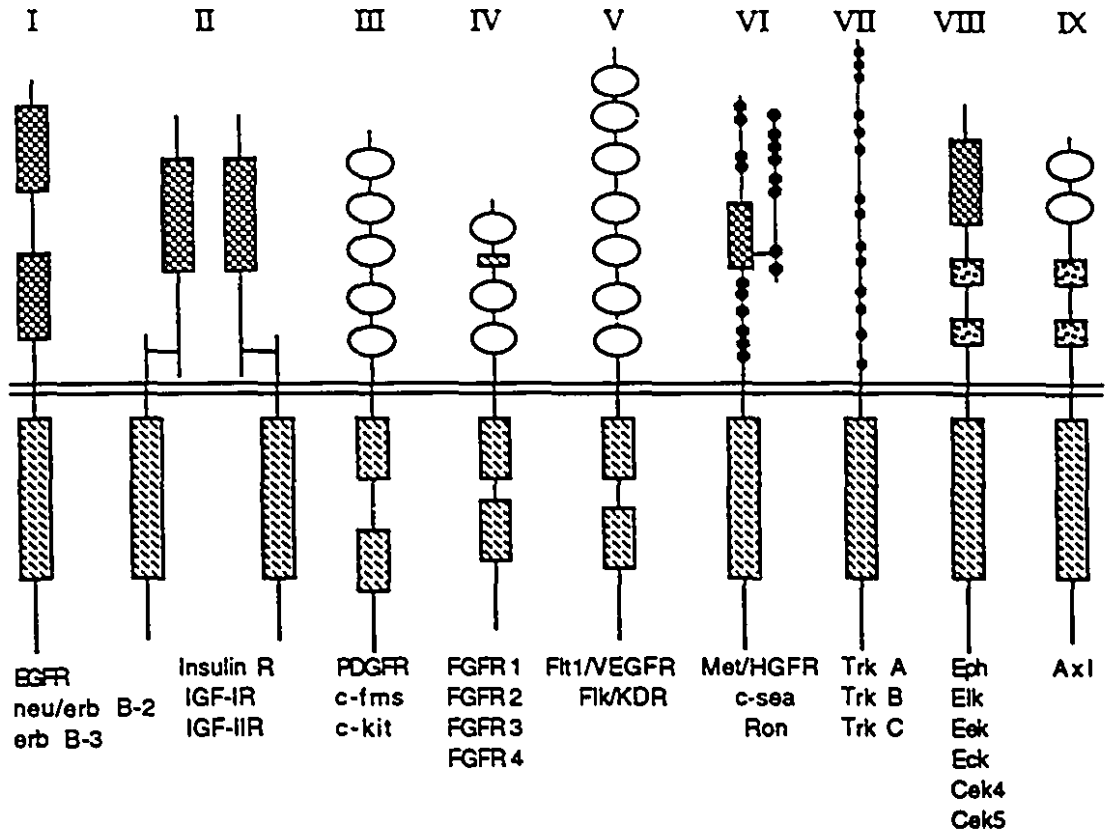

Figure 1. Classification of receptor tyrosine kinases. The following structure features are identified: transmembrane domains, = , tyrosine kinase domains, ▨ , cysteine-rich domains, ▩ , immunoglobulin-like domains, ○ , acid box domain, ▭ , fibronectin type III domains, ▨ , and conserved cysteine residues, ● .

presence of two cysteine-rich sequence repeats within the extracellular domain. A regulatory C-terminal extension is located downstream of the catalytic kinase domain which contains the major autophosphorylation sites. The cysteine residue spacing and similarity of interspaced sequences are strictly conserved in the cysteine-rich repeat. The ligands which bind with high affinity to the EGFR include EGF (Cohen et al., 1962), transforming growth factor-α (TGF-α), (Derynck et al., 1984), amphiregulin (Shoyab et al., 1989), the scahwannoma-derived growth factor (SDGF) (Kimura, et al., 1990), cripto and heparin binding EGF (HB-EGF) (Higashiyama et al., 1991). The ligands for the neu receptor are

the recently cloned neu differentiation factor (NDF) and the neu/erb B ligand growth factor (NEL-GF) (Wen et al., 1992; Huang and Huang, 1992).

Subclass II includes the insulin receptor (IR), the insulin-like growth factor-1 receptor (IGF-1R), and insulin-related receptor (IRR). In this class the receptor precursor is cleaved to form a disulfide-linked heterodimeric $\alpha\beta$ structure. Addition disulfide bonds are subsequently formed between the two α chains of the $\alpha\beta$ heterodimer generating the mature $\alpha_2\beta_2$ receptor. The α subunits form a pocket for ligand binding, whereas the β subunits transverse the plasma membrane and transduce the signal through the kinase activity situated in the cytoplasmic domain. One cysteine rich repeat is present in each α subunit. Ligands for this class of receptor include, insulin, IGF-1, IGF-2 and several as yet unidentified ligands.

Subclass III is represented by the two types of PDGF receptor (α and β) , the colony-stimulating factor-1 (CSF-1) receptor and the stem cell factor receptor (c-kit). These monomeric receptors are characterized by the presence of five immunoglobulin-like repeats in the extracellular domain and a non-catalytic kinase-insert region, that varies in length between receptors. The ligand for PDGFR has been found in at least three dimeric forms AA, AB, and BB. The α receptor binds to both AA and AB forms, whereas the β receptor only binds to the BB form (Heldin, 1992).

Subclass IV encompasses the family of fibroblast growth factor receptors (FGFR). The structure of this family is similar to that of the PDGFR subclass except they have three immunoglobulin-like repeats and an acid box in the extracellular domain.

Subclass V members are structurally related to members of the PDGF receptor family. However, they have seven instead of five immunoglobulin-like repeats within the extracellular domain. The vascular endothelial cell growth factor (VEGF) receptor is representative of this group.

Subclass VI includes the *met*/HGF/SF receptor. The structure of these receptors is similar to that of the insulin receptor with a single αβ heterodimeric molecule as a mature form. Additional members of this family include recently discovered receptors, the c-Sea proto-oncogene and the Ron gene (Huff et al., 1993; Ronsin, et al., 1993).

Subclass VII receptors are monomeric with an extracellular domain containing scattered conserved cysteine residues and an uninterrupted cytoplasmic tyrosine kinase domain. The *trk* gene family encodes the A, B and C forms of the receptor which bind to a family of ligands including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic-3 (NT-3), NT-4, and NT-5.

Subclass VIII includes monomeric receptors with a cysteine-rich sequence repeat and two fibronectin type II domains located in the extracellular region. Representatives are Eph, Elk, Eck, Eek, Erk, and Cek4 and Hek receptors which are encoded by distinct genes. The ligands which bind to these receptors remain unidentified.

Subclass IX is represented by the Axl receptor. It is also a monomeric receptor with two immunoglobulin-like repeats and two fibronectin sequence repeats within its extracellular region. The ligand that binds to the AxlR is unknown.

In summary, the presence of conserved sequences within the extracellular domains of each receptor subclass suggests that these domains are functionally significant. The similarity between different subclasses appears to have originated as a result of gene sequence duplication. As more receptors are being characterized and sequenced, more structural variations are likely to be observed leading to an increase in the number of receptor subclasses, such as the Ros and Ret, Tie-2 RTKs which do not appear in Fig. 1.

1.3. Biochemical properties of RTK subdomains.

Following ligand binding, the enzymatic activity of the RTK is increased. This activity promotes receptor dimerization and transphosphorylation (or autophosphorylation) of intracellular tyrosine residues (Ullrich and Schlessinger, 1990), which in turn triggers the association of cellular substrates with the receptor (Fig. 2) (reviewed, Panayotou and Waterfield, 1993).

A number of studies, using chimeric receptors to investigate the functional domains of RTK's, suggest that ligand binding specifically is dependent on the extracellular domain of the receptor, whereas signal transduction is a function of the cytoplasmic domain and requires a kinase activity (Lax et al., 1989; Lev et al., 1990). EGF binding was mapped to the area between the two cysteine-rich regions in the extracellular domain of the EGF receptor by cross-linking ^{125}I-EGF to a chimeric human-chicken EGFR (Lax et al., 1989). These data suggests that the cysteine-rich regions may provide a specific recognition pocket for ligand binding. Perhaps the immunoglobulin-like sequences characteristic of the extracellular domains of certain RTK's could serve the same function. The understanding of specific interactions between growth factors and their RTKs will be greatly enhanced by the use of x-ray crystallography to determine the three dimensional structure of both ligand and receptor binding pocket.

All growth factor receptors contain a transmembrane domain which is characterized by a stretch of hydrophobic amino acids which form an α-helix. The mechanism by which this short sequence transmits a signal from the extracellular domain to the intracellular domain remains unknown. However, a point mutation (valine to glutamic acid) in the transmembrane region of the Neu receptor onco-protein results in full activation of the transforming potential (Bargmann et al., 1986). Because this mutation enhances receptor

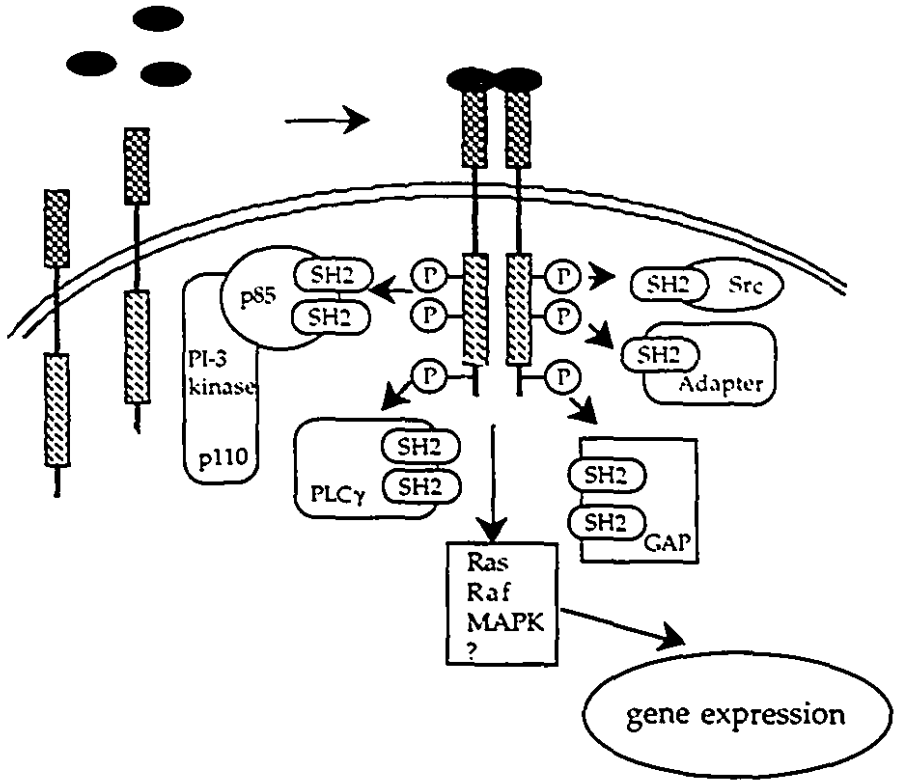

Fig. 2. A model for RTK activation and assembly of signaling complexes. Ligand binding results in RTK dimerization and autophosphorylation on several tyrosine residues, and creates biding sites for signaling proteins containing SH2 domains

oligodimerization, it has been proposed that the transmembrane domain of RTKs may have a role in facilitating receptor dimerization (Weiner et al., 1989).

The role of the cytoplasmic domain in signaling by RTKs has been intensively investigated. The kinase domain is highly conserved, not only within the RTK family, but also between other tyrosine kinases, and to a lesser extent between serine- and threonine- specific kinases (Hunter and Cooper, 1985). Within the kinase domain, a consensus sequence, GlyXGlyXXGlyX(15-20)Lys (Yarden and Ullrich, 1988; Ullrich and Schlessinger. 1990) is present and provides the binding site for adenosine triphosphate (ATP), permitting the catalytic transfer of γ-phosphate from ATP to tyrosine residues of protein substrates, both in the RTK itself as well as in various cellular substrates. Mutation of the Lys

residue in EGFR and PDGFR results in a complete loss of kinase activity, loss of coupling to cellular substrates, a lack of receptor internalization and cellular response (Chen et al., 1987; Escobedo et al., 1988). The same effects also appear following microinjection of anti-phosphotyrosine antibodies. These observations suggest that phosphorylation of the receptor on tyrosine residue(s) itself and/or of cellular substrates is essential for the function of the RTK (Glenney et al., 1988). In the kinase domain of the RTK, there are one or more conserved tyrosine residues, which serve as targets of autophosphorylation (or transphosphorylation of homodimers of the RTK), and regulate the catalytic kinase activity of the RTK (Tornqvist and Avruch, 1988). These tyrosine residues represent the major autophosphorylation sites.

1.4. RTKs and cellular substrates in signal transduction.

Following ligand binding, activated RTKs are associated directly with a subset of cytoplasmic signaling molecules (Fig. 2) (Panayotou and Waterfield, 1993). Depending on the type pf receptor these may include c-Src, GTPase-activating protein (GAP), Grb-2, tyrosine phosphatases (e.g. PTP-1C) or phospholipases e.g., phosphatidylinositol 3 (PI-3)-kinase, phospholipase C-γ (PLC-γ) (Pawson and Gish, 1992). These substrates subsequently transmit signals to cellular compartments further downstream. The association between signaling proteins and RTKs is strictly dependent upon phosphorylation of tyrosine residues adjacent to short sequence motifs which provide binding sites in the receptor for proteins containing Src homology 2 (SH2) domains. SH2 domains have been identified in a diverse group of proteins, some of which contain enzymatic activity, such as PLC-γ, GAP, PTP-1C and c-Src. Others, such as Nck, v-crk, Grb 2 and p85 of PI-3 kinase (Pawson and Gish, 1992) only serve as adapter proteins that couple RTKs to effector proteins. Binding of SH2 domains to phosphorylated RTKs is thought to provide a common mechanism by which

diverse enzymatic and regulatory proteins can interact specifically with RTKs and thereby couple growth factor stimulation to multiple intracellular signaling substrates such as p21 Ras, Raf-1 kinase, MAP kinase, and S6 ribosomal kinase (Pawson and Gish, 1992). SH2 domains are often accompanied by a stretch of conserved sequences which leads to a specific assembly of cellular substrates to the RTK. Besides the SH2 domain, most of these signaling molecules contain one or more SH3 domains that are involved in binding proteins containing proline rich repeats (Cicchetti et al., 1992). One of these proteins is the SOS guanine nucleotide exchange protein that is involved in activation of Ras proteins (Li et al., 1993; Rozakis-Adcock et al., 1993). Ultimately, the activation of various signaling pathways mediated by RTKs and associated proteins leads to changes in gene expression.

1.5. Oncogenes and RTKs.

The eukaryotic genome harbors a large number of genes, termed proto-oncogenes whose mutation or aberrant expression can lead to malignancy. Proto-oncogenes encode a diverse set of proteins, polypeptide growth factors and cytokines, transmembrane growth factor receptors, intracellular transducers of growth factor responses, and nuclear transcription factors that mediate growth factor induced gene expression (Cantley et al., 1991).

Many proto-oncogenes exhibit protein kinase activity and are thought to induce malignant growth through modification of the activity. Constitutive activation of the tyrosine kinase activity is an absolute requirement for activation of the oncogenic potential of RTKs (reviewed in Rodrigues and Park, 1994). One manner by which RTKs are activated is through structural changes, which alter the receptor conformation leading to constitutive kinase activity in the absence of growth factor stimulation. Mutations of this type include the point mutation in the transmembrane domain of the Neu oncogene, deletion of specific regulatory

domains of RTKs (e.g. v-erbB, v-fms, v-kit), and chromosomal rearrangements of RTKs (e.g. tropomyosin-trk, TPC-ret, tpr-met). Another mechanism of activation involves the aberrant expression of the growth factor and/or RTK. Gene amplification and overexpression of RTKs have been shown to be associated with a number of tumors. For example, the EGFR gene is amplified in glioblastomas and several squamous carcinomas (Libermann et al., 1985, Yamamoto et a., 1986) and the HER2 gene is amplified in adenocarcinomas and in mammary tumors (King et al., 1985; Yorkota et al., 1986; Zhou et al., 1987; Slamon et al., 1987). It appears that establishment of an autocrine growth response, whereby a tumor cell both expresses and is stimulated by, a mitogenic growth factor and its receptor, will allow cells to become less dependent on the environment and contribute to cellular transformation. When cDNA expression vectors encoding the relevant growth factor are introduced into growth factor-dependent hematopoietic and fibroblast cells, the cell lines become both growth factor independent and tumorigenic (Heldin and Westemark, 1989). Moreover, analysis of the expression of mRNA and protein for PDGF A chain and the PDGF α receptor in sections of human glioblastomas, provides *in vivo* evidence for an autocrine loop which contributes to tumor progression (Hermansson et al., 1992).

2. RTKs mediate diverse biological functions in a cell-type specific manner.

2.1 Introduction.

Stimulation of cells by growth factors that bind to and activate RTKs is followed by rapid alterations in gene expression, cellular metabolism, and cytoskeletal architecture. These in turn evoke cellular responses such as proliferation, differentiation and migration. The precise cellular response to RTK activation is dependent upon the identity of the RTK and the nature of the cell being stimulated. Although a number of RTKs are activated in a broad range of

cells, many are transcribed in a tissue-specific or developmentally regulated fashion and exert a function only in specific target cells.

2.2. The family of Trk RTKs play a role in survival of specific neurons.

An example of a tissue-specific and developmentally regulated RTK is the family of Trk RTKs which plays a role in the survival and/or differentiation of specific neurons. The Trk A RTK preferentially binds to NGF and is involved in sympathetic and sensory neurons (Table 1 Bradshaw et al., 1993; Raffioni et al., 1993; Davies 1994), whereas Trk B is the preferred receptor for BDNF and NT-4, and plays a role in nodose neuron and some sensory neurons, as well as neurons of the central nervous system (CNS) (Table 1, Squinto et al., 1991; Klein et al., 1991). The trk C RTK transcript is widely distributed in the CNS and is the receptor for NT-3 (Lamballe et al., 1991). The identification of unique receptors for different neurotrophins, as well as the potential for cross-reactivity, provides a basis for the variety of target cell responses. Although characterization of specific functions for members of the Trk family is on-going, it is clear that this family of RTKs may mediate the survival and differentiation signals in neurons of the central and peripheral nervous system (PNS).

2.3. The pleiotropic functions of RTKs are governed by interactions with specific growth factors.

An interesting feature of several RTKs is their broad target-cell specificity. For example the EGFR is expressed in preimplantation embryos as early as the eight-cell stage in mice and is present in various cell types dervived from all three germ layers, except hematopoietic cells (Wiley et al, 1992). TGF-α is expressed in preimplantation mouse embryos (Roppolee et al., 1988), in several tissues of day 9 and day 10 embryos (Wilcox and Derynck, 1988), as well as in a variety of adult tissues. TGF-α binds to the EGFR and governs several important

biological processes including cell proliferation (Derynck et al., 1984), keratinocyte migration (Barrondon and Green, 1987), angiogenesis (Schreiber et al., 1986), wound healing (Schultz et al., 1987) and cell differentiation (Luetteke et al., 1993). On the other hand, EGF is expressed in tissues of newborn mice (Rall et al., 1985), and involved in skin differentiation, accelerated eye opening and tooth eruption via the EGFR (Cohen et al., 1962). These data demonstrate that the EGFR mediates pleiotropic functions by binding to different ligands and that the specificity is regulated through production of the ligand in a time dependent and/or tissue specific manner.

Table 1. The responsibility of neuronal cells to neurotrophins and their receptors.

	NGF	BDNF	NT-3	NT-4& -5
PNS				
Sensory neurons (Dorsal root ganglia)	+	+	+	+
Sympathetic neurons	+	-	+/-	-
Nodose neurons	-	+	+	+
PC12 cells	+	-	-	-
CNS				
Cholinergic neurons	+	+	+/-	ND
Dophaminerigic neurons	-	+	+	ND
Retinal ganglion cells	-	+	ND	ND
Receptor Binding				
Trk A	+	-	+	+
Trk B	-	+	+	+
Trk C	-	-	+	?

The *steel* factor (known as alternatively mast cell growth factor, stem cell factor and kit ligand) encodes protein isoforms, one of which is a cell surface

protein, and one of which is cleaved to yield a soluble factor (Flanagan,, 1991). The membrane associated *steel* factor, binds to and activates the Kit RTK and acts as an adhesion molecule to mediate cellular aggregation. This protein is necessary for normal mammalian development (Flanagan, 1991). The *Steel* mutant mouse, that produces only a soluble form of *Steel* factor, is unable to support normal development of melanocytes, hematopoietic and germ cells, suggesting that the cell surface form of *Steel* is indeed important *in vivo*. In culture, the cell surface form of *Steel* factor is required for the survival of germ cells (Dolci et al., 1991). Thus, the functions of Kit RTKs are regulated throught interactions with different isoforms of *steel* factor.

2.4. Activation of RTKs is specifically regulated.

Several growth factors, including members of the EGF family, are synthesized as inactive membrane-spanning precursor molecules which are released following proteolytic cleavage. Access to the target receptor by the ligand can therefore be restricted, and be dependent upon the presence of proteolytic enzymes. For example, the release of TGF -α depends on the presence of tissue-specific elastase-like proteolytic enzymes (Derynck et al., 1984; Lee et al., 1985). The cellular response to RTK stimulation can also be regulated in a concentration-dependent manner by the growth factor. For example, fibroblasts respond to high concentrations of FGF-1 by a chemotactic response, and to low concentrations with a mitogenic response (Linemeyer et al., 1987).

An alternative mechanism whereby RTK mediated cellular responses are restricted depends on the cellular localization of both the RTK and its specific ligand, i.e. whether the mode of action is endocrine, paracrine or autocrine. Both the PDGF A chain and the PDGF α receptor mRNA and protein are expressed in the same cell at the two-cell and blastocyst stages during development, which implies an autocrine mode of action (Palmieri et al., 1992). In contrast, early post-

implantation mouse embryos express PDGF-A chain mRNA in embryonic ectoderm lining the ectoplacental cavity, while the PDGF α receptor mRNA is localized to the mesodermal layers of both embryonic and extraembryonic membranes, suggesting a paracrine mode of action. These observations provide evidence for a developmental shift from an autocrine to a paracrine fashion of PDGF A and PDGF α receptor interaction.

Yet another mechanism by which activation of RTKs is regulated involves the interaction of growth factors with both high and low affinity receptors, examplified by the family of FGFs and their specific receptors. To date, four human genes encoding high affinity FGFRs (K_d of -10^{-11}M) have been identified (Klagsbrun and Baird, 1991). Cell surface heparin sulfate proteoglycans (HSPGs) function as low affinity receptors (K_d of -2×10^{-11}M) for basic FGF (Moscatelli, D., 1987) and are essential for FGF binding to its high affinity recptor (Yayon et al., 1991; Rapraeger et al., 1991). HSPGs modulate both the binding of basic FGF to its high affinity receptor and determine the specificity of basic FGF cell signaling (Nurcombe et al., 1993). A number of other growth factors, including EGF, PDGF, VFGF, acidic FGF, GM-CSF, and HGF/SF have been shown to bind to heparin. The EGFR shows a much higher affinity for cripto and heparin binding (HB)-EGF than for EGF. In addition, HB-EGF is more potent than EGF as a mitogen in macrophage-like cells (Higashiyama et al., 1992).

2.5. The specific cellular response mediated by the RTK relies on downstream signals.

Different RTKs elicit distinct biological responses in the same target cell. One example of this diversity is demonstrated by primary neuronal cells or rat Pheochromocytoma (PC12) cells in response to EGF or FGF. EGF treatment leads to stimulation of cell growth and proliferation (Huff et al, 1981), whereas, stimulation of PC12 cells with either acidic or basic FGF (Togari et al, 1985;

Wagner, 1991), or NGF (Huff et al., 1981), leads to growth arrest and differentiation into a sympathetic neuronal phenotype. Thus, the same cell can elicit distinct physiological responses to different RTKs. Moreover, a single RTK can elicit distinct biological responses in different cell types. The TrkA RTK induces neuronal differentiation in PC12 cells, whereas it induces mitogenesis in fibroblasts (Pulciani et al., 1982; Martin-Zanca et al., 1986). The specific cellular response mediated by an RTK is dependent on its association with distinct downstream substrates.

Despite the diversity of responses mediated by RTKs, there is a great degree of similarity in the intracellular signaling pathways used by these proteins. For example, the mitogenic response mediated by the EGF RTK and the induction of differentiation by the TrkA and FGF RTK in PC12 cells are both activated by the Ras pathway (Chao, 1992). On the other hand, the three TrkC isoforms, p145TrkC K1, TrkC K2, TrkC K3, which have the same amino acid sequences except 14 and 25 additional amino acid residues located downstream of the putative autophosphorylation sites (Lambelle et al, 1993), elicite different biological responses upon binding to the NT-3. All isoforms induce mitogenesis of NIH3T3 cells, however, only TrkC K1 induces neuronal differentiation of PC12 cells. TrkC K1, but not TrkC K2 or K3, is able to phosphorylate PLC-γ and PI-3 kinase. These observations suggest that the different biological responses are probably mediated by the association of the receptor with different downstream substrates.

3. RTKs are important for cell differentiation and development.

3.1. Introduction

The high level of conservation of RTK-related sequences during eukaryotic evolution suggests that RTKs may be essential in regulating many of

the signals that control cell growth, cell movement and cell differentiation during embryonic development. The mapping of spontaneous mouse mutants within receptors and their ligands has provided some insight into the function of RTKs and growth factors in embryonic development. However, a more informative method for determining the function of RTKs during development involves gene "knock-outs" by the technique of homologous recombination in embryonic mouse stem cells and the subsequent generation of chimeric mice with a null mutation. Research in this area usually starts with a description and characterization of the temporal and spatial expression of the gene. Table 2 summarizes some of the data characterizing RTK expression during development. Together with the use of *in vitro* model systems and/or cell lines, a knowledge of the expression pattern of RTKs and growth factors will elucidate their biological functions during development.

3.2. Murine developmental loci for mutated growth factors and RTKs

Recent advances in gene cloning and mapping techniques have facilitated the identification of candidate genes for existing mouse mutants (Table 3). On the other hand, analysis of the connection between the phenotypic properties of the mutant locus and the known biochemical properties and/or expression pattern of a closely linked gene provides a basis to further investigate the relationship between the mutant locus and the candidate gene. Phenotypic analysis of these loci has yielded clues to the biological properties of the gene product. Gene mapping also provides information on the molecular defects underlying certain mutations. The importance of RTKs in cell differentiation and development was marked by the discovery that the c-Kit RTK is allelic with the murine *dominant-white spotting* locus (W) on chromosome 5, whereas its ligand was mapped to the *steel* (Sl) locus on chromosome 10 (Geissler et al., 1988; Copeland et al., 1990; Witte, 1990). Mutations at the W locus or the Sl locus results in a similar

Table 2. RTK expression during development

Gene	Occurrence	Function
EGFR	Ectoderm, mesoderm and endoderm from the 8 cell stage and throughout out embryogenesis	Mitogenesis, differentiation and survival.
IGF-IR	Morula stage. inner cell mass and trophoectoderm	Mitogenesis, metabolism and differentiation.
IGF-II R	2-cell stage	Mitogenesis, metabolism and differentiation.
FGFR	Mesenchymal cells, somites, neuronal and epithelial cells	Mitogenesis.
PDGFR	Mesenchymal and glial cells, smooth muscle, placental trophoblasts. PDGFRα: 2 cell stage; PDGFβ: 8 cell stage	Mitogenesis, wound healing, and hemotaxis.
c-fms	Myeloid/macrophages and extraembryonic cells	Mitogenesis and Maturation.
c-kit	Hematopoietic cells, germ cells and melanocytes	Mitogenesis, migration, differentiation and survival.
trk	Neural crest cells in CNS and PNS D9.5 embryo	Survival.
VFGFR	Endothelial cells	Mitogenesis.
tie-2	Endothelial cells of blood vessels D8.5 embryo	Mitogenesis and invasion.
flk-1	Endothelial cells of heart and blood vessels. D6.5 embryo	Differentiation.
ros	Epithelial cells in kidney, intestine, lung. D11.5 embryo	Morphogen and differentiation.
neu/erb B2	Epithelial cells, schwan and glial cells	Mitogenesis or differentiation.
met	Epithelial cells, melanocytes, and hepatocytes	Mitogenesis, migration, morphogen and toxic.
ret	Epithelial cells, neural crest cells D8.5 embryo	Mitogenesis, migration, differentiation and survival.

phenotype including severe macrocytic anemia, mast cell deficiency, sterility, and coat color abnormalities. Despite these similarities, transplantation, tissue chimeras, and *in vitro* co-culture assays have all confirmed that the cellular basis of W and Sl mutations are distinct. W mutations result in a defect within stem cells of hematopoietic and melanogenic lineages, whereas the Sl defect lies within the microenvironment of these cells. The presence of different W mutants provides insight into the mechanism of c-Kit RTK mediated signal transduction. The dominant mutations, W^{37}, W^{v}, W^{41} and W^{42} are the result of missense mutations in the kinase domain of the c-*kit* coding sequence (W^{37} at position 582' W^{v} at 660, W^{41} at 831, and W^{42} at 790), and consequently affect the tyrosine kinase activity of the c-Kit RTK in these mice (Nocka et al., 1990; Morrison-Graham and Yoshiko, 1993). The kinase negative mutants W^{37}, W^{42}, and W which harbors a 78aa deletion including the transmembrane domain are embryonic lethal suggesting that the kinase domain of c-Kit RTK is essential for murine development. The diverse phenotypic abnormalities of the W mutations allow us to understand how c-Kit RTK signals mediate diverse biological responses including cell migration, proliferation, differentiation and survival of primordial melanocytes, germ cells, and pluripotent hematopoietic cells.

Another example of a RTK involved in cell growth and development is the c-*fms* gene product, a receptor for the macrophage growth factor or colony-stimulating factor 1 (CSF-1) (Yoshida et al., 1990). The spontaneous mouse mutation *osteopetrosis* (op) on chromosome 3 results in loss of function of the *CSF-1* gene. The mutant phenotype is characterized by severe bone abnormalities resulting from the defective growth of osteoclasts from precursor macrophages and (Marks and Lane, 1976; Yoshida, et al., 1990; Begg et al., 1993). A reduction in macrophage numbers has also been noted in this mutant, which is a consequence of a defective microenvironment rather than an intrinsic stem cell defect, suggesting that CSF-1 and the CSF-1R (c-*fms*) play an important role in the

regulation of proliferation, differentiation, and survival of the macrophage lineage. (Wilktor-Jedrzejczak et al., 1982; Marks, 1984). Another mutation carrying of osteropetrotic phenotype, *microphthalmia* (*mi*), encodes a protein necessary for transducing signals mediated by either the c-Kit or the c-fms RTK (Dubreuil et al., 1991). This provides evidence that mutation of genes affecting the common receptor signal transduction pathway will lead to similar phenotypes

In addition, it has recently been shown that the PDGF-α RTK gene is partially deleted in the *Patch* (*Ph*) mutant locus, which maps adjacent to the W locus on chromosome 5 (Stephenson et al., 1991). The *Ph* heterozygote shows hair coloration anomalies implying defects in melanoblasts. Whereas, *Ph* homozygote mice die on day 11 of gestation following the development of various anatomic abnormalities, including craniofacial and cardiac outflow tract separation defects, which correspond to the mapped distribution of the PDGF-α receptor. These defects in the *Ph* mice suggest that the PDGF-α RTK may be involved in the early development of visceral endoderm and mesoderm derivatives and later in neuronal and mesodermal development. (Schatteman et al., 1992; Orr-urteger et al., 1992; Morrison-Graham et al., 1992).

3.3. Functional analysis of growth factors and RTKs by homologous recombination.

To date, only a very small proportion of the available mouse mutants have been characterized at the molecular level, which limits the speed with which the *in vivo* biological properties of the signal transduction network can be investigated. The genetic redundance also modified the phenotypes of the mouse mutants and therefore creates difficulties in understanding the molecular mechanisms of signal transduction *in vivo*.. However, the generation of mice carrying mutations within specific genes by homologous recombination in

embryonic stem cells will allow the analysis of mutants that do not exhibit an overt phenotype (Table 3), and possibly lead to an understanding of the functional compensation in the intact animal.

Recently, two groups described mutant mice in which the TGF-α gene was disrupted by homologous recombination (Luetteke et al., 1993; Mann et al., 1993) The receptor for TGF-α is the EGFR which is expressed in a wide variety of adult tissues as well as throughout embryogenesis. TGF-α has been shown to be one of the most important growth factors, being involved in the development of many tissues. Surprisingly, mice carrying a null mutation of TGF-α gene did not exhibit any remarkable disorders except defects in skin architecture manifested in disorganized follicles, wavy hair and curly whiskers; as well as corneal inflammation in some of the older mice. This phenotype is remarkably similar to that of the spontaneous mouse recessive mutant *waved-1* (wa-1), located on chromosome 6. Northern analysis revealed reduced expression of TGF-α in wa-1 mice. Crosses between wa-1 homozygotes and TGF-α deficient mice confirmed that the TGF-α and wa-1 are allelic. Another phenotypically similar mutation, *waved-2*, which is cell autonomous within the hair follicle, originally mapped near the EGFR on chromosome 11. A point mutation was subsequently found in the wa-2 mice that locates within the kinase domain of the EGFR, which significantly reduced its kinase activity as well as its binding to exogenous substrates. This indicates that wa-2 and EGFR may be allelic (Luetteke et al., 1994). These results suggest that TGF-α and EGFR may play a pivotal role in determining skin architecture and in regulating hair development. The mild phenotypes of the TGF-α deficient mice and wa-2 mutant also suggest that there is functional compensation in the intact mouse.

In mice homozygous for a mutation in the Trk B RTK (Klein et al., 1993) two of five trk B transcripts, encoding the full length p145 Trk B RTK and the non-catalytic transmembrane p95 Trk B protein, are absent. Heterozygotes

Table 3. Mouse mutations of RTKs and their ligands

Gene	Locus	Phenotypic Abnormalities
c-kit	W (domiant white spotting)	Melanoblasts, hematopoietic and germ cells.
Kit ligand	Sl(steel)	Melanoblasts, hematopoietic and germ cells.
CSF-1 (c-fms ligand)	op(osteopetrosis)	Osteoclasts and macrophages.
PDGF-αR	Ph(patch)	Melanoblasts and hematopoietic cells, D11 lethal.
EGFR	wa-2(waved-2)	Outer root sheath of active hair follicle, curly hair and whiskers.
TGF-α	wa-1(waved-1) and Knock -out	Inner root sheath of active hair follicle, curly hair and whiskers.
trk B	Knock-out	Die prenatal day 1, loss of fatal motoneuron and neurons in spinal cord, trigeminal & dorsal root ganglia.
BDNF	Knock-out	Most die prenatal day 2, loss of cranial and spinal sensory neurons, develop ataxia.
p75 NGFR	Knock-out	Loss of sensory neuron of foot pads.
trk A	Knock-out	Neuronal loss of trigeminal, sympathetic and dorsal root ganglia, decrease in the cholinergic and basal forebrain projection to the hippocampus and cortex.
NGF	Knock-out	Perinatal loss of sensory and sympathetic neurons, develop basal forebrain cholinergic neurons.
IGF-1R	Knock-out	Dwarf, Neonatal lethal due to respiratory failure, organ hypoplasia including muscle, delay in ossification, and abnormalities in CNS and epidermis.
IGF-1, IGF-2	Knock-out	Dwarfs.

appear to develop normally, whereas homozygous mutants die by prenatal day 1, appearently due to poor innervation of the motoneuron system, a consequence of cell death of some neurons in the central (facal motor neurons and spinal cord) and peripheral (trigeminal and dorsal root ganglia) nervous system resulting in feeding problems in the neonate. This data suggests that the p145 Trk B RTK is involved in the ontogeny of the nervous system, however the role of p95 Trk B remains unclear. However, mice lacking the Trk A RTK (Smeyne et al., 1994) die within one month of birth due to extensive neuronal loss in trigeminal, sympathetic and dorsal root ganglia, as well as to a decrease in the cholinergic and basal forebrain projection to the hippocampus and cortex. These observations suggest that Trk A is the primary mediator of the trophic actions of NGF *in vivo*. To more fully understand the roles played by different isoforms of the Trk RTK family in development, mutations must be generated in other members of this family.

The IGF-1 and IGF-2 have dual functions, acting both as mitogens in response to growth hormone stimulation, and as differentiation factors in embryonic development. Targeted mutagenesis of IGF-2 revealed that the growth-promoting function of IGF-2 is restricted to embryogenesis (DeChiara et al., 1990, 1991). The IGF-2 gene is parentally imprinted such that the maternal allele is silent in most tissues. Therefore, the heterozygotes carrying the paternally derived IGF-2 mutation gene and mice homozygous for the IGF-2 mutation (IGF-2, -/-) are phenotypically indistinguishable. These mutants are viable and fertile, but proportional dwarfs, with a body weight 60% of that the wild type littermates (DeChiara et al., 1990, 1991). Mice carrying the null mutation for IGF-1 (IGF-1 -/-) are also dwarf (60% of the normal size) and either die shortly after birth or survive to adulthood depending on the genetic background (Liu et al., 1993). The IGF-1R mutant (IGF-1R -/-) exhibits a more severe growth retardation (45% of the normal size), and dies at birth of

respiratory failure accompanied by organ hypoplasia, developmental delay in ossification and abnormalities in the central nervous system and epidermis (Liu et al., 1993). Analysis of mouse embryos carrying null mutations of IGF-1, IGF-2 and IGF-1R, alone or in combination, revealed that between embryonic days 11 and 12.5, the IGF-1R serves as the RTK for *in vivo* mitogenic signaling for IGF-2 only. From day 13.5 onward, the IGF-1R interacts with both IGF-1 and IGF-2, while IGF-2 recognizes an additional uncharacterized receptor which plays an exclusive role in placental growth (Baker et al., 1993). These studies provide a good evidence that generation of mice carrying null mutations in genes for growth factors and their receptors is an effective means by which interactions between those growth factors can be examined during development.

The loss-of function Ret RTK mutant dies soon after birth, showing renal agenesis or severe dysgenesis, and lacking enteric neurons throughout the digestive tract (Schuchardt et al., 1994). Expression of the Ret RTK was first detected in day 8.5 embryos, in the developing nervous (sensory, autonomic and enteric ganglia) and excretory system (Wolffian duct and ureteric bud epithelium) (Pachnis et al., 1993). It was recently demonstrated that mutations in the Ret RTK is associated with four human syndromes (van Heyningen, 1994), Hirschsprung's disease (absence of autonomeric ganglia cells) (Edery et al., 1994), and different types of thyroid carcinoma; FMTC, MEN2A and MEN 2B (Mulligan, et al., 1993; Hofstra et al., 1994; Romeo et al., 1994).

3.4. Other developmental loci in the downstream signaling of RTKs.

In addition to mouse mutations in genes encoding RTKs, other mutations have been mapped to downstream cytoplasmic kinases and transcriptional factors. Analysis of these mutants will shed light on the understanding of RTK mediated signal transduction pathways in the regulation of mammalian development. A tyrosine phosphatase PTP-1C, which has been shown to

associate with the c-Kit RTK through its SH2 domain following stimulation with Steel factor, has been mapped to the mouse recessive mutant, *motheaten* (*me*) and *viable motheatent* (me^v) (Shultz et al., 1993; Tsui et al., 1993; Yi and Ihle, 1993). Loss of PTP-1C activity in these mutant mice causes severe autoimmunity and immunodeficiency (Green et al., 1989).

Another mouse mutation resulting in immunodeficiency has been mapped to a cytoplasmic tyrosine kinase, Bruton's tyrosine kinase (Btk), which belongs to the Src kinase family and is a homolog of human X-linked agammaglobulinemia (XLA) gene (Rawlings et al., 1993; Thomas et al., 1993; Vetrie et al., 1993). A point mutation in the XLA gene will lead to an accumulation in the bone marrow of pre-B cells, which fail to expand and mature into functional B cells (Tsukada, et al., 1993; Vetrie et al., 1993). Intensive efforts have been made to knock-out Src kinase family members by homologous recombination. Targeted disruption of the c-Src kinase has been accomplished resulting in homozygous mutants which develop osteopetrosis, a bone modeling disorder due to a defect in osteoclast function (Lowe et al., 1993). Homozygous mutant mice lacking the *csk* gene, which is thought to downregulate Src, die between day 9 and day 10 of gestation with a complex phenotype which includes neural tube defects. Cells derived from these embryos have a dramatic increase in the kinase activity of Src and Fyn (Imamoto and Soriano, 1993). Loss of Fyn causes defects in the hypocampus. (Appleby et al., 1992) and absence of Yes shows no discernible phenotype. In an Lck knock out mouse thymocytes are blocked early in the developmental program and very few T cells emerge in the periphery (Molina et al., 1992). The phenotypes of these mutant mice were somehow milder than that expected. This further suggests a functional compensation for the Src kinase family which is shown to associate with activated RTKs.

4. RTKs and invertebrate development.

4.1 Introduction.

During the last decade, genetic and molecular analyses of developmental processes in invertebrates have greatly expanded our understanding of the molecules that participate in the regulation of development. Studies of a number of invertebrate developmental regulatory genes led to the striking discovery that genes involved in signal transduction pathways including RTKs, are highly conserved in mammals, *Drosophila* and *C. elegans*, supporting the importance of these genes for normal development and cell functions. Studies of RTK homologues in organisms that are more amenable to genetic and developmental analysis, such as *Drosophila* and *C. elegans*, will facilitate our understanding of the RTK family in the regulation of mammalian growth and development. The genetic tools available for research in *Drosophila* and *C. elegans* provide a powerful approach for the dissection of components of the signal transduction pathway.

4.2. *Drosophila* EGFR RTK.

The first RTK gene isolated from *Drosophila* was the DER (*Drosophila* EGF receptor homolog) gene, which exhibits a high degree of homology to two related mammalian RTK genes, the EGF receptor and the *neu* gene (Livneh et al., 1985; Bargmann et al., 1986b). The DER protein is present in two forms, differing at the extreme amino terminus as a result of alternative splicing (Schejter et al., 1986). The DER gene is transcribed during all stages of development (Lev et al., 1985). No maternal transcript is found, but transcription can be detected in early embryos. At the larval stage transcripts are restricted to the dividing diploid cells of the imaginal discs and to the brain cortex, suggesting a role in cell proliferation. In the adult, DER mRNA is detected in the nervous system and in

follicle cells of ovaries (Schejter et al., 1986; Kemmermeyer et al., 1987). By screening the recessive lethal mutants defective in the DER locus and using genetic complementation assays, it has been shown that DER mutations are allelic to the lethal *faint littleball (flb)* mutation, Flies carrying the *flb* mutation which exhibit a defect in the development of the larval cuticle and have no *in vitro* DER kinase activity (Nusslein-Vollhard et al., 1984; Schejter et al. 1989). The homozygous DER mutants also develop other embryonic lethal phenotypes, including deterioration of head structure, absence of ventral denticle bands, defective germband retraction, and collapse of the central nervous system along the ventral midline. These observations indicate that the DER protein plays a role in a number of different tissues during development. Another known mutant, *Ellipse* is a dominant mutation of the DER gene encoding a partially active receptor and develops rough eyes with a reduced number of ommatidia (Baker and Rubin, 1989), suggesting that DER is also involved in the transmission of inhibitory signals, which determine the proper spacing of photoreceptor clusters in the eye imaginal disc of larva. A maternal ventralizing locus, *torpedo* is shown to be allelic to *faint littleball* and has a deletion in the DER gene (Price et al. 1989). The *torpedo* homozygous females are sterile due to the development of ventralized eggs and egg shells. These results suggest that the DER gene plays a central role in the *Drosophila* embryonic development and that DER mutants may display different phenotypes which are associated with different aberrations in the EGF RTK signal transduction pathway.

4.3. *Drosophila Torso* RTK.

Another example of a RTK in *Drosophila melanogaster* is that of *torso*, the PDGF-β receptor homolog, which is a maternal gene and responsible for the proper differentiation of the terminal, nonsegmented regions of the embryo (Sprenger et al., 1989; Perrimon, 1993). In *Drosophila*, patterning along the antero-

posterior axis requires the activity of a three determinant system that controls anterior, posterior, and terminal portions of the body pattern (reviewed in Nusslein-Volhard, 1991). The anterior patterning is mainly determined by the activity of a homeodomain gene product, the *bicoid* protein (bcd), whose transcripts are localized initially at the anterior pole of the egg during oogenesis and then translated following fertilization to give rise to a gradient of the protein (Driever and Nusslein-Volhard, 1988). Posterior patterning is specified by the graded distribution of a transcriptional factor, *nanos* (Perrimon, 1993). The *torso* RTK protein accumulates uniformly along the entire surface of the embryo, but is activated only in the vicinity of the pole in response to an extracellular ligand generated at each end of the egg (Klinger et al., 1988; Stevens et al., 1990). Thus the *torso* RTK is a good candidate gene for involvement in determination of the pattern of two noncontiguous embryonic domains, the anterior and posterior terminus. *Torso-like* , a potential *torso* ligand, is diffused in the perivitelline space in the absence of the receptor (Sprenger, 1992; Casanova and Struhl, 1993). This indicated that the *torso* receptor has a dual function which is to restrict diffusion of the ligand and to transduce the spatial signal.

4..4. *Sevenless* RTK and eye development in *Drosophila* .

Drosophila sevenless is most similar to the vertebrate c-Ros RTK. It can induce a non-neuronal lens-secreting cell to develop into a specific photoreceptor cell, R7 (Hafen et al., 1987). Therefore, *sevenless* is required to specify cell fate in the developing eye (Rubin, 1991). Activation of *sevenless* by the ligand, *bride of sevenless* (boss) which is expressed on the membrane of a neighboring R8 photoreceptor cell, results in transduction of a signal which specifies the R7 cell fate (Harte et al., 1990). There is also a soluble form of boss which acts as an antagonist of the *sevenless* receptor (Hart et al., 1993). However, the differentiation of R7 cells requires contact between R8 and R7 cells. Cone cells,

which also express *sevenless*, do not develop as R7-like cells because they are spatially restricted from contact with the ligand-expressing R8 cell. Flies carrying loss-of-function alleles of *sevenless* have an inactive kinase with a single amino acid substitution at the ATP-binding site. These flies fail to induce R7 development and transform the R7 cell to non-neuronal cone cell phenotype, but have no other obvious defects (Basler and Hafen, 1988). A gain-of-function *sevenless* mutation generated by overexpressing a truncated *sevenless* protein in cells which normally express *sevenless*, initiates neuronal development and results in the formation of multiple R7-like photoreceptor per ommatidium in a dose dependent manner (Basler et al., 1991). Constitutive activation of *sevenless* does not lead to the proliferation of cells but to the transformation of R7 cell fate.

4.5. Other RTKs in *Drosophila* .

The use of veterbrate RTKs as hybridization probes, under conditions of low stringency has enabled the isolation of more RTK homologs from the *Drosophila* genome. These homologs include the *Drosophila* Insulin receptor (DIR) (Petruzzelli et al., 1986), fibroblast growth factor receptor (DFGFR) (Glazer and Shilo, 1991) and the Dtrk gene (Pulido et al., 1992). The DIR mRNA is detected most abundantly in 8- to 12- hour embryos and the tyrosine kinase activity is greatest during embryogenesis, suggesting a role for DIR during embryogenesis (Petruzzelli et al., 1985, 1986). In contrast to the diversity of the vertebrate FGFR family, there is only a single homologue of *Drosophila* FGFR, which encodes three immunoglobulin (Ig)-like domains in the extracellular region and a split kinase in the cytoplasmic region (Glazer and Shilo, 1991). Expression of DFGFR is detected at all stages of development, and the mRNA and protein are localized to the developing tracheal system, and the delaminating midline glial and neural crest cells. In *breathless* (*btl*) embryos, homozygous for a deletion which includes the DFGFR locus, the initial formation of the tracheal pits is not affected, but the

extension of tracheal cell processes is disturbed, leading to a block in formation of the elaborate tree structure. A chimeric receptor of *torso* and DFGFR kinase domain is shown to partially rescue the migration defects in the *btl* embryos (Reichman-Fried et al., 1994), suggesting the DFGFR protein may play a role in tracheal cell migration.

The *Drosophila trk* gene has an overall structural similarity to the vertebrate *trk* gene family (Pulido et al., 1992). The *Dtrk* protein contains six Ig-like domains in its extracellualr region, whereas the mammalian trk has only two. This Ig-like domain is not only present in the RTK family, such as PDGFR, c-kit, c-fms and FGFR (Yarden and Ullrich, 1988), but is also found in cell adhesion molecules including N-CAM (Cunningham et al., 1987), as well as the membrane-associated fasciclin and neuroglian (reviewed in Grenningloh et al., 1991). *Dtrk* is involved in cell adhesion, which specifically activates its tyrosine kinase activity (Pulido et al., 1992). It is not known if the adhesive property of the *Dtrk* is mediated by the Ig-like domain. However, the widespread occurrence of this Ig-like region in RTKs suggests a possible role for this domain in cell-cell interactions. The expression of *Dtrk* is detected mainly in the nervous system, including neurons and fasciculating axons (Pulido et al., 1992). These observations raise the possibility that tyrosine phosphorylation may play an important role in cell-cell communication during development of the nervous system.

4.6. RTK in C. *elegans* development.

The genetic basis of cell signaling pathway has been intensively studied in the nematode C. *elegans*, a lower metazoan with a normally invariant developmental fate. The gonadal anchor cell sends an inductive signal which causes three to six vulval precusor cells to adopt the vulva fate. The *let*-23 gene product is required for this induction, and is proposed to be functional in the

presumptive vulval cells (Sternberg and Horvitz, 1991). Molecular analysis has revealed that the *let*-23 gene encodes a RTK of the EGF receptor family (Aroian et al., 1990). Gain-of function mutations of *let*-23 cause a multivulval phenotype (Ferguson et al., 1985; 1987). Loss-of function mutations result in a vulvaless phenotype, lethality in the first larval stage, hermaphrodite sterility, improper differentiation of the male tail and improper development of the posterior ectoderm, suggesting that activation of the *let*-23 RTK is involved in the differentiation of multiple cell types (Ferguson et al., 1985; 1987, Aroian and Sternberg, 1991). The domains involved in these differrent functions of the *let*-23 RTK have recently been mapped to different portions of the c-terminus of *the protein* (Aroian et al., 1994). The *let*-23 acts upstream of *let-60*, a homologue of the mammalian *ras* gene, in the vulval determination pathway (Han, 1990). *Lin*-3, which represents a structural homolog of EGF/TGF-α is expressed in the anchor cell and acts upstream of *let*-23 (Hill et al., 1992). Overexpression of *lin*-3 produces a dominant, multivulva phenotype dependent on *let*-23 function. This identifies the *lin*-3 gene product as a likely ligand for the *let*-23 receptor. While vulval development is not essential in C. *elegans*, null mutations in *let* 23 are lethal, suggesting that *let*-23 functions in other essential signaling pathways.

4.7. Where is the specificity?

Detailed genetic analyses in *Drosophila* and C.*elegans* have ascertained that signals mediated by DEGFR, *torso*, *sevenless* and *let*23 are all transmitted through the Ras (or *let-60* in C.*elegans*) pathway which activates a downstream protein-serine/threonine kinase cascade that controls cellular differentiation (Table 4). Activation of the Ras protein, p21 Ras1, is a crucial early event in the *sevenless* signaling pathway, and constitutive activation of p21 Ras1 is sufficient to induce all of the biological effects mediated by *sevenless* (Fortini et al., 1992). It is shown that a *Drosophila* SH2-SH3 containing protein *drk*, a homolog of the *Sem-5* protein

of *C.elegans* and mammalian GRB2, is essential for signaling by the *sevenless* receptor (Olivier et al., 1993, Simon et al, 1993). GRB2 serves as an adaptor protein, which binds to a tyrosine phosphorylated receptor via its SH2 domain and the *Sos* (*son of sevenless*) guanine nucleotide-releasing protein through its SH3 domain, thereby coupling the receptor to Ras activation.

Table 4. Signal transduction pathways of several RTKs

RTK	Torso	Sevenless	DER	let--23	human PDGFR
SH2 adaptor	Drk	Drk	Drk	sem-5	GRB-2
PTPase	corkscrew	?	?	?	SHPTP2
RasGNRFs	Sos	Sos	Sos	?	Sos
Ras	Ras1	Ras1	Ras1	let-60	Ras
GAP	?	GAP1	?	?	GAP
Ser/Thr kinase	Raf	Raf	Raf	lin-45	Raf1
Thr/Tyr kinase	Dsor1	Dsor1	?	?	MAPK(Mek)
Ser/Thr kinase	rolled	rolled	rolled	?	MAP
Nuclear factors	bicoid/?	sina/yan	?	?	?
Biological activities	terminal differentiation	R7 induction	Epidermal differentiation	Vulval differentiation	Proliferation

The *torso* pathway involves the protein tyrosine phosphatase, *corkscrew*, (*csw*) and the *Draf* serine/threonine protein kinase, which eventually controls the expression of the transcription factors,*tailless* and *huckebein* (Melnick et al., 1993). These transcription factors are activated by the *bicoid* protein (bcd) and repressed by the *torso* RTK at the anterior pole of embryos (Ronchi et al., 1993). Mutagensis screening for dominant suppressors of a *torso* gain-of-function allele revealed that Ras-1, *Sos* and *Dsor1* (Mammalian MAP kinase kinase, Mek homolog) are

also involved in the *torso* pathway (Doyle et al., 1993). Injection of an activated p21 v-Ras protein can rescue the maternal-effect phenotypes of both *torso* and *csw* null mutations, and this rescuing effect is dependent on the presence of *Draf* activity. Thus, p21ras/Ras1 operates upstream of *Draf* in the signal transduction pathway (Lu et al., 1993). In *C.elegans*, it has been shown that *lin-3* signals mediated by *let-23*, are also transmitted by *sem-5*, then *let-60* and *Lin-45* (mammalian Raf homolog) (Dickson and Hafen, 1994). Together, these observations indicate that common substrates are present in the signal transduction pathways of the DER, *torso*, *sevenless* and *let-23* receptors.

The main components of the Ras signalling pathway are not only conserved in worms and flies, but also in mammalian RTKs (e.g. PDGFR and EGFR). It is intriguing that multiple biological functions are mediated by the different RTKs yet all are ultimately transmitted by the Ras pathway. Even though the qualitative output of all RTKs is similar, it is possible that the RTK acts as on/off switch and that specific responses can be elicited in a time and tissue dependent manner, and reflect the presence of a variety of transcriptional regulatory elements.

5. *Met* encodes the RTK for hepatocyte growth factor and scatter factor.

5.1. The discovery of the *tpr-met* oncoprotein and the *met* proto-oncogene.

The *met* gene was originally identified as an activated oncogene, *tpr-met*, in a N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treated human osteogenic sarcoma cell line (HOS) by its ability to transform NIH3T3 mouse fibroblasts (Cooper et al., 1984). The oncogenic *met* was generated by a chromosomal rearrangement that fuses *tpr* sequences from chromosome 1 to the *met* proto-oncogene locus on chromosome 7 (Fig. 3B) (Park et al., 1986). Sequence analysis of cDNA revealed that human *met* encodes a growth factor receptor tyrosine

kinase protein (Park et al., 1987). Subsequent cloning of the mouse *met* proto-oncogene (Chan et al., 1988; Iyer et al., 1990) revealed an overall sequence homology of greater than 88.1% between human and mouse cDNAs. As demonstrated by Northern hybridization studies, *met* mRNA is expressed ubiquitiously in both embryonic and adult mouse tissues (Chan et al., 1988; Iyer et al., 1990).

Biosynthetic analysis and pulse chase experiments revealed that the *met* gene encodes a 150 kDa precursor, which is subsequently glycosylated to yield a 170 kDa precursor protein which is localized to the cell surface as a monomeric molecule (Giordano et al., 1989b; Rordrigues et al., 1991). The mature *met* RTK is composed of a transmembrane 145 kDa β subunit which is disulfide-linked to an intracellular 50 kDa α subunit to form an heterodimeric form of 190 kDa (Fig. 3B) (Gonzatti-Haces et al., 1988). A consensus sequence (KKRKQ) for proteolytic cleavage, similar to that found in the insulin receptor, is present in the extracellular domain of the *met* RTK (Park et al., 1987). It has been demonstrated that the α and β subunits of the *met* RTK were generated from proteolytic cleavage of the p170 kDa glycosylated precursor (Rordrigues et al., 1991). In the GTL-16 human gastric carcinoma cell line, in which the *met* gene is amplified and overexpressed (Giordano et al., 1989a), two truncated forms of the *met* protein were identified, a 140 kDa transmembrane form and a 130 kDa soluble protein, both of which contain the α subunit and a truncated β subunit (Fig. 3A. Giordano et al., 1989b). Whether these *met* RTK truncated forms are present due to a deficiency in the enzymatic activity required for the cleavage event in this cell line remains to be determined. However, multiple *met* transcripts of 8, 7, 5, 3 and 1.6 kb have been detected in a variety of human epithelial cell lines (Park et al, 1986, Giodano et al, 1989a). The identification of cDNA sequence corresponding to these alternatively spliced forms reveals that they may encode proteins with predicted structures as illustrated in figure 3B (Rodrigues and Park, 1993).

However, the biological activities of these *met* RTK isoforms have not yet been determined.

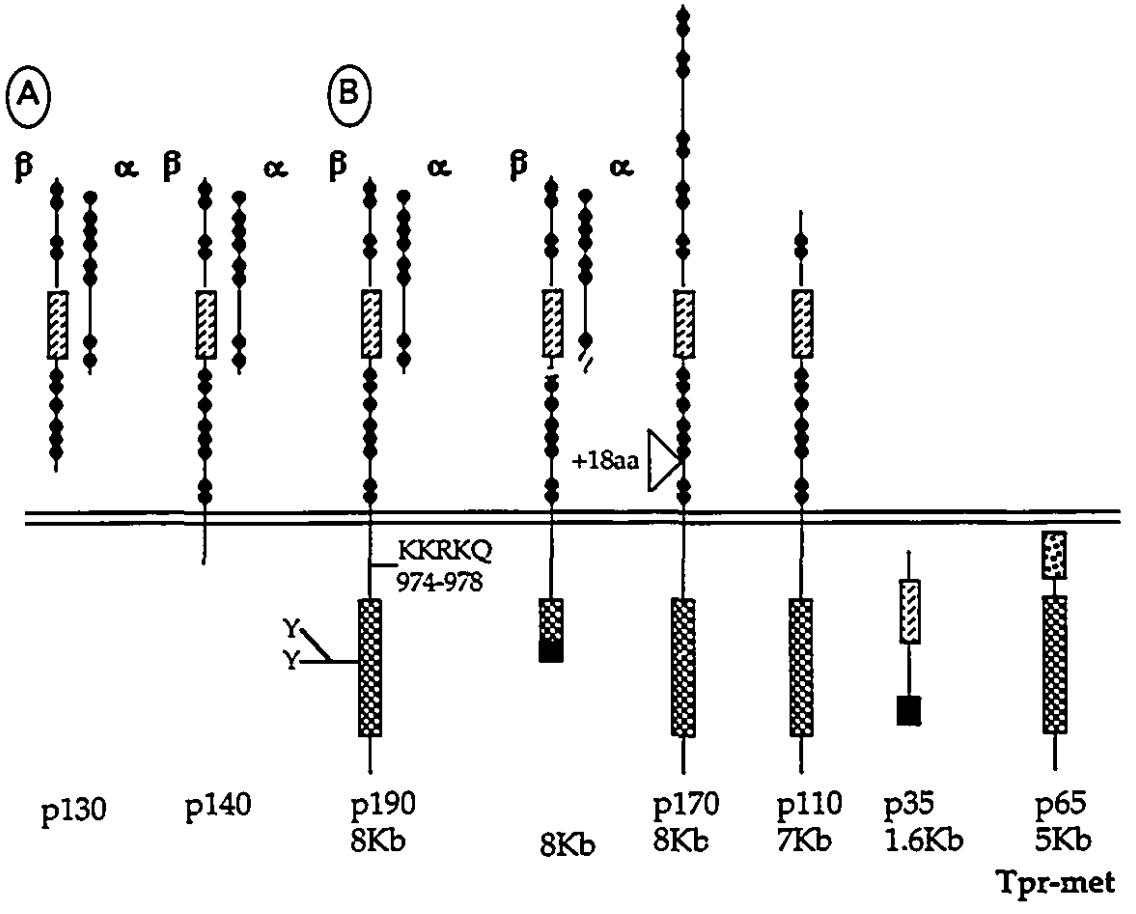

Figure 3. Structures of Met isoforms. A: Two Met isoforms are generated by proteolytic cleavage. B: Predicted structures of various Met transcripts

Biochemical analysis has revealed that the protein products of both the *met* proto-oncogene (p145 *met*) and the *tpr-met* oncogene (p65 *tpr-met*) can undergo autophosphorylation on tyrosine residues in an *in vitro* immunecomplex kinase assays (Gonzatti-Haces et al., 1988). However in the majority of cell lines studied, the p145*met* β subunit is normally phosphorylated only on serine and threonine residues, and is only phosphorylated on tyrosine residues following stimulation

with ligand (Giordano et al., 1989a; Bottaro et al., 1991). In common with other activated kinase oncogenes, p65 *tpr-met* is constitutively phosphorylated on tyrosine residues (Gonzatti-Haces et al., 1988), and this is required for the transforming potential of the *tpr-met* oncogene (Rodrigues et al., 1991).

5.2. A multifunctional cytokine, HGF/SF is the ligand for the *met*RTK.

The ligand for the *met* RTK was identified as the hepatocyte growth factor/hepatopoietin A (HGF) by coimmunoprecipitation and covalent cross-linking studies (Bottaro et al., 1991; Naldini et al., 1991a). Treatment of intact target cells with HGF rapidly induced tyrosine phosphorylation of the 145 kDa β subunit of the *met* RTK. HGF was first purified from human and rabbit plasma and primary rat platelets on the basis of its ability to stimulate mitogenesis in rat hepatocytes, and therefore was thought to be a humoral mediator of liver regeneration (Zarnegar et al., 1989). HGF was also isolated from a variety of fibroblast cells, and shown to stimulate DNA synthesis in epithelial and endothelial cells, as well as in melanocytes and keratinocytes in culture. However, the inability of HGF to stimulate mitogenesis in the fibroblasts from which it was derived suggests that HGF is a paracrine factor involved in mesenchymal-epithelial cell interactions (Rubin et al., 1989). Another soluble factor, scatter factor (SF), was purified from human embryonic fibroblast cells, MRC 5 (Stoker, et al., 1987; Gherardi et al., 1989), as well as from cultured bovine calf aorta and human iliac smooth muscle cells (Rosen et al., 1989). SF causes scattering of epithelial cells by inhibiting the formation of junctional complexes, increasing local motility, and inducing the conversion of epithelial cells into a more fibroblastic phenotype (Gherardi et al., 1989).

cDNA cloning and biochemical analysis identified HGF and SF as the same molecule. This heterodimeric heparin-binding protein consists of a heavy (α) and a light (β) polypeptide chain with apparent molecular weights of 65 kDa

and 35 kDa, respectively (Tashiro et al., 1990; Naldini et al., 1991b; Weidner et al., 1991; Bhargava, et al., 1992). The α chain contains four kringle domains, similar to those present in plasminogen. However, unlike plasminogen HGF/SF does not show any proteolytic activity. Molecular analyses have demonstrated that the kringle structures are required for protein-protein interaction and that their deletion results in complete loss of binding of HGF/SF to the *met* RTK and subsequent loss of its biological activities (Matsumoto et al., 1991). HGF/SF is ubiquitiously expressed in adult rat tissues in three splicing isoforms, 6, 3 and 1.3 kb (Chan et al., 1991). The 6 and 3 kb transcripts encode the same protein, differing only in the 3' untranslated region, whereas the 1.3 kb transcript encodes a protein lacking the last two kringle domains and having no mitogenic activity. This small truncated molecule appears to compete with HGF/SF for binding to the *met* RTK, and thereby specifically inhibits HGF/SF induced mitogenesis (Chan et al., 1991; Lokker et al., 1993; Mizuno et al., 1994). These observations suggest that the same gene encodes both a growth factor and its specific antagonist.

In addition to its functions as a mitogen and motogen, HGF/SF can also behave as a morphogen. Treatment of canine kidney epithelial (MDCK) cells cultured in a collagen matrix with HGF/SF will induce their polarization and results in formation of branching tubules (Montasino et al., 1991a& b). HGF/SF has also been shown to strongly inhibit the proliferation of a large number of tumor cells including those from hepatomas, melanomas, and squamous cell carcinomas, both *in vitro* and *in vivo* (Tajima et al., 1991; Shiota et al., 1992). It has therefore been suggested that HGF/SF is identical to the tumor cytotoxic factor, which has been purified from human alveolar macrophages, blood monocytes and embryonic lung fibroblasts, IMR-90 cells (Higashio et al., 1990; Shima et al., 1991). However, using chimeric receptors, it has been demonstrated that all of the cellular responses to HGF/SF are specifically mediated through the kinase

domain of the *met* RTK (Weidner, et al., 1993; Komada and Kitamura, 1994; Zhu et al., 1994).

5.3. Involvement of HGF/SF and the *met* RTK in embryonic development.

Grafting of HGF/SF enriched cells and beads onto the chick egg induced formation of a secondary primitive streak (Stern et al., 1990), suggesting a possible function of HGF/SF in embryonic development. *In situ* hybridization studies showed that the *met* RTK is expressed predominantly in epithelial cells (Soneberg, 1993; Yang and Park, 1994). Since HGF/SF is expressed predominantly by embryonic fibroblast cells in culture, this suggests that HGF/SF may play an important role as a paracrine mediator in the interactions of epithelial and mesenchymal cells. When HGF/SF and the *met* receptor are coexpressed in NIH3T3 cells, the cells become tumorigenic in nude mice (Rong et al., 1992) and show conversion from a mesenchymal cell to an epithelial phenotype (Tsarfaty et al., 1994). During embryonic development, the conversion between mesenchymal and epithelial cells occurs as early as the gastrulation stage when the mesodermal cells are formed by ectodermal cell dissociation, migration and conversion. Determination of the time at which HGF/SF and *met* RTK are first expressed during development will allow us to gain a better understanding of the physiological function of both ligand and receptor.

It has been demonstrated that the aberrant expression of the *met* RTK results in deregulation of cell growth. Besides the chromosomal rearrangement of *tpr-met*, overexpression of the *met* RTK is also suggested to contribute to cellular transformation in the GTL-16 cell, a human gastric carcinoma cell and in colorectal carcinomas (Ponzetto et al., 1991; Liu et al., 1992). Furthermore, the autocrine expression of HGF/SF and the *met* receptor is shown to induce transformation of NIH3T3 cells (Rong et al., 1992) and is present in bronchial epithelial and many non-small cell lung carcinomas (Tsao et al., 1993).

5.4. Signal transduction mediated by the *met* RTK.

To understand the signal transduction pathways mediated by RTKs, one must first characterize the association of cellular substrates with the ligand-activated RTKs. For HGF/SF and the *met* RTK, this issue is complicated by divergent responses of different cell types following the same ligand-receptor interaction. Proteins associated with the *met* RTK pathway to date are the p85 subunit of PI3-kinase and MAP kinase (ERK2) in human keratinocytes and melanocytes under conditions in which proliferation took place in response to HGF/SF stimulation (Graziani et al., 1991; Faletto et al., 1993). It appears that the MAP kinase tyrosine phosphorylation is absent from MDCK cells, which scatter rather than proliferate following treatment with HGF/SF (Chatani et al., 1992). Association of other substrates including PLC-γ, Ras GAP, Src, Fyn have been demonstrated by direct coimmunoprecipitation or by the use of *in vitro* association assays (Bardelli et al., 1992; Faletio et Al., 1993). It was recently demonstrated that one tyrosine residue (489) at the carboxyl terminus of *trp-met* is essential for efficient transformation by the *tpr-met* oncoprotein, and mutation of this tyrosine impairs the association betwwen *tpr-met* and PI-3 kinase, Grb2, and an unidentified protein of 110kDa (Fixman et al., 1994). In addition, changes in cytoskeletal elements have been documented in response to HGF/SF stimulation with a marked reduction in the number of F-actin stress fibers being found in highly motile cells (Dowrick and Warn, 1990). However, how HGF/SF and *met* RTK trigger these different signaling pathways and generate distinct biological responses remains unknown.

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Chapter II Expression of the Hepatocyte Growth Factor/Scatter Factor Receptor Tyrosine Kinase Is Localized to Epithelia In The Adult Mouse

Preface

To maintain the tissue homeostasis and normal development, the expression of RTKs is regulated in a tissue and cell type specific manner. One approach to understand the normal physiological function of a RTK is to identify the cell type specific expression. Previous reports had demonstrated that *met* R is expressed ubiqutiously in many adult mouse tissues by Northern hybridization studies, and little was know about its normal physiological function. To investigate if *met* R is expressed in cells common to all of these tissues, I was interested to study the cell-type specific expression of *met* R in adult mouse tissues by the *in situ* hybridization technique. The following manuscript describes that, the *met* R is expressed preferentially in surface and tubular epithelial cells and in hepatocytes of resting liver, as well as in granulosa cells of the developing and mature follicles. The *met* R was identified to be the receptor for hepatocyte growth factor and scatter factor and mediates cell proliferation, migration, tubulogenesis of epithelial and endothelial cells in culture. Therefore, the observation of *met* R expression in epithelial cells *in vivo* supports a role of *met* R in epithelial cell growth and tissue organization. The biological implications of such an observation are discussed

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Abstract

The *met* proto-oncogene receptor tyrosine kinase has been identified as a receptor for hepatocyte growth factor (HGF)/scatter factor (SF). HGF/SF is a multifunctional cytokine that stimulates mitogenesis, dissociation and motility of a broad spectrum of epithelial and endothelial cells in culture, promotes the progression of carcinoma cells to a more invasive phenotype and acts as a morphogenic factor for tubular epithelia. HGF/SF is predominantly expressed by mesenchymal cells whereas the *met*/HGF/SFR is predominantly expressed by epithelial and carcinoma cells in culture. We have shown by Northern analyses that the *met*/HGF/SFR is expressed in many adult mouse tissues. To elucidate the normal physiological role for the *met*/HGF/SFR and the possible pathological consequences of deregulation of this pathway, we have examined the expression of the *met*/HGF/SFR in adult mouse tissue by *in situ* hybridization. We show that the *met*/HGF/SFR is generally expressed in epithelia, including hepatocytes, epithelial cells that line the proximal and distal convoluted tubules of the kidney, epithelia of stomach, oesophagus, uterus, lung and skin, as well as in granulosa cells of maturing and mature oocytes. By reverse transcriptase polymerase chain reaction amplification we show that the HGF/SF gene is expressed at low levels in many of these tissues. Our data support a possible role for the *met*/HGF/SFR in epithelial cell growth and tissue organization.

Introduction

Many growth factor receptors possess an intrinsic tyrosine specific protein kinase activity and play an important role in the transduction of growth regulatory signals across the plasma membrane (Yarden & Ullrich, 1988). Growth factors and their corresponding receptor tyrosine kinases (RTK) are frequently expressed in a cell type specific fashion and are involved in controlling many physiological processes such as cell mitogenesis, movement and differentiation (Forrester et al., 1992). The critical nature of these interactions for normal development is manifested by the fact that mutation or altered expression of RTKs or their ligands can lead to abnormal cell growth, differentiation and development (Klein et al., 1993; Luetteke et al., 1994).

The human *met* proto-oncogene is a member of the RTK gene family (Park et al., 1987) and has been identified as the receptor for hepatocyte growth factor/scatter factor (HGF/SF) (Bottaro et al., 1991; Naldini et al., 1991b). The major *met* proto-oncogene product is synthesized as a single chain precursor of 170kDa that is cleaved to yield a transmembrane β subunit of 145kDa, containing an intracellular kinase domain (Tempest et al., 1986; Gonzatti-Haces et al., 1988; Giordano et al., 1989a, b) and an α subunit that remains extracellular (Rodrigues et al., 1991). The α and β chains are associated through disulfide bonds and form the mature heterodimeric *met* receptor product of 190kDa which upon binding HGF/SF becomes phosphorylated on tyrosine residues on the β chain (Bottaro et al, 1991; Naldini et al., 1991b, c). Autophosphorylation is involved in the activation of the catalytic activity of the $p190^{met}$ kinase (Naldini et al., 1991a) and this event is essential for biological activity of the receptor (Zhu et al., 1994).

HGF/SF is predominantly expressed by mesenchymal and stromal cells in culture (Stoker & Perryman, 1985; Rosen et al., 1989) and is now recognized as a multifunctional cytokine that is a potent mitogen for a broad spectrum of epithelial and endothelial cells in culture (Matsumuoto & Nakamura, 1993). In

addition, HGF/SF stimulates the dissociation and motility of epithelial and endothelial cells (Stoker & Gherardi, 1991), promotes the progression of carcinoma cells to a more invasive phenotype (Weidner et al., 1990) and acts as a morphogenic factor that stimulates the formation of branching tubules from tubular epithelial cells (Montesano et al., 1991). The unique biological activities of HGF/SF and its role as a paracrine effector for epithelial cells, suggests that it may be an important molecule involved in epithelial-mesenchymal interactions that occur normally during tissue organization, embryogenesis and organogenesis.

Stimulation of the *met*/HGF/SFR is sufficient to mediate the pleiotrophic activities of HGF/SF in epithelial cell lines in culture (Weidner 1993; Komada & Kitamura, 1993; Zhu et al., 1994) and suggests that the *met* /HGF/SFR would mediate the various physiological responses to HGF/SF in the animal. The *met*/HGF/SFR is expressed in the majority of epithelial and carcinoma cell lines in culture (Park et al., 1986; Gonzatti-Haces et al., 1988; Prat et al., 1991; Di Renzo et al., 1991) and is expressed in many adult tissues in mouse (Chan et al., 1988; Iyer et al., 1990) and human (Prat et al., 1991). The *met*/HGF/SFR was initially identified as an activated oncogene (Cooper et al., 1984) and overexpression of the murine or human *met*/HGF/SFR with its corresponding ligand is oncogenic for NIH3T3 fibroblasts (Iyer et al., 1990; Rong et al., 1992). Thus the biological activities of HGF/SF and the observation that the *met*/HGF/SFR is amplified and/or overexpressed in human carcinoma cell lines in culture (Giordano et al., 1989, Halaban et al., 1992; Ponzetto et al., 1991) and in human tumors (Liu et al., 1991; Tsao et al., 1993; Ponzetto et al., 1991) suggests that the *met*/HGF/SFR and its ligand may be involved in pathological processes such as cancer and metastasis.

To investigate the possible physiological functions of the *met*/HGF/SFR and the possible pathological consequences of deregulation of this pathway, we

have examined the expression of the *met*/HGF/SFR in adult mouse tissues by *in situ* hybridization. We show that *met*/HGF/SFR is generally expressed in epithelia including; hepatocytes, epithelial cells that line the proximal and distal convoluted tubules of the kidney, epithelia of stomach, oesophagus, lung, uterus and skin, as well as in granulosa cells of maturing and mature oocytes. Furthermore, by reverse transcriptase polymerase chain reaction amplification we show that the HGF/SF gene is expressed at low levels in many of these tissues. Our data support a possible role for HGF/SF and the *met*/HGF/SFR in epithelial cell growth and tissue organization.

Materials and methods

Plasmids

As probes for hybridization studies, a 0.5kb EcoRI fragment (p*met* SC1) derived from the 5'-portion, or a 2.1kb EcoRI fragment (p*met* SC3) from the 3' portion of the murine *met* cDNA (pcD) (Iyer, et al., 1990) were subcloned into the pBluescript KSII$^+$ vector (Stratagene). The rat HGF/SF 1.4kb EcoRI cDNA fragment including the 3'-portion of α subunit, β subunit and a portion of 3'-untranslated region was subcloned in a pBluescript SK (-) (pRBC-1) (Tashiro et al., 1990).

RNA Extraction and Northern Hybridization

Tissues were freshly dissected from the F1 mice of a B6 and C3 cross. RNAs from various tissues were prepared following the protocol of Chomczynski and Sacchi 1987. Northern transfers were performed as described in Park et al., 1986. For each sample 40μg of 1 x Poly A selected RNA was electrophoresed in a 1.2% agarose, 6% formaldehyde gel and blotted onto a Hybond N membrane. Filters were prehybridized in 0.5M Na_3PO_4, 1mM EDTA, 1% BSA, 7% SDS and 200μg salmon sperm DNA at 60^{0}C for 1-4 hour, then hybridized in 30% formamide, 0.2M Na_3PO_4, 1mM EDTA, 1% BSA, 7% SDS and 200μg salmon sperm DNA and 10^6cpm/ml of radiolabeled probe of 1.4kb rat HGF/SF 3'cDNA or 2.1 EcoRI *met* 3' cDNA fragment. Filters were washed once in 2 x SSC, 0.1% SDS at room temperature for 20 min, then twice for 30 min in 0.1 x SSC, 0.1% SDS 60^{0}C.

In situ hybridization

Tissues were dissected from the F1 mice of a B6 and C3 cross and then either stored in liquid Nitrogen or directly mounted with OCT freezing medium. Sections were cut with a cryostat (Leitz) at 10μ and placed on the poly-lysine

coated slides. Sections were briefly air dried, then fixed in fresh 4% paraformaldehyde in PBS for 15 min at RT and washed twice in 1 x PBS, treated with proteinase K (10µg/ml) in 0.1M Tris-HCl pH 7.5, 50mM EDTA, 2mM $CaCl_2$ at 37°C for 20 min followed by incubation in 0.1M Triethanolamine containing 0.25% acetic anhydride for 10 min. For control slides, RNA was digested by treatment with 2µg/ml of RNase A and RNase T1 in 0.1M Tris, pH 7.5, 50mM EDTA and 4mM NaCl at 37°C for 1h prior to the proteinase K treatment. Slides were washed in 3 x PBS for 5min, 1 x PBS for 5' and 0.2% glycine in PBS for 10 min, then prehybridized in a buffer containing 50% deionized formamide, 5 x SSC, 5 x Denhart's solution, 250µg/ml tRNA and 200µg/ml salmon sperm DNA at 45°C for at least 1h. Sections were dehydrated in alcohol prior to hybridization.

Antisense and sense riboprobes were generated to the 0.5kb p*met* SC1 or 1.5kb p*met* SC2 cDNA fragment by *in vitro* transcription using digoxigenin-UTP following the manufacturers instructions (Boehringer Mannheim; Munjaal, 1990). Probes were diluted in prehybridization mix at 5ng/µl, and 40µl of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, then washed twice with 2 x SSC at 45°C for 15 min, and treated with 2µg/ml of RNase A at RT for 15 min. Slides were then washed once with 0.2 x SSC and twice with 0.1 x SSC at 45°C for 15 min each and blocked with 2% normal sheep serum and 0.3% triton X-100 in buffer 1 (100mM Tris-HCl. pH 7.5, 150mM NaCl) for 30 min. To detect *met*/HGF/SFR specific hybrids, slides were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (1 to 5,000 dilution in buffer 1) for 30 min, then washed twice (15 min each) with buffer 1 and rinsed in buffer 3 (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM $MgCl_2$). The hybrids bound to anti-digoxigenin antibody can be visualized by a colour reaction containing 337.5µg/ml nitrobluetetrazolium salt (NBT), 175µg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.24mg/ml Levamisole in buffer 3 and colour was allowed to develop for 12 hours in the

dark. The reaction was terminated by incubation with 100mM Tris-HCl, pH 8.0, 1mM EDTA for 5 min. Slides were dehydrated, incubated in xylene, mounted with Permount and stored at 4°C in the dark. Slides were viewed and photographed on a light microscope.

Reverse transcriptase PCR amplification

Total RNA (5µg), isolated from different tissues, was resuspended in 16µl of DEPC-treated water, denatured in 8µl of 0.1M methyl mercury and neutralized in 6µl of 0.7M β-mercaptoethanol. First strand cDNA was synthesized using 1.25µg random hexamers (Pharmacia) as primers as described in Park et al., 1987. The reaction was terminated, and cDNA was extracted with phenol/chloroform and resuspended in 100µl dH_2O. To amplify an HGF/SF specific product of 278 bp, two oligonucleotide primers P1 (5'-483 CCATGAATTTGACCTCTATG 503-3') and P2 (5'-761 ACTGAGGAATGT-CACAGACT 740-3') were selected (Tashiro et al., 1990). The PCR reaction contains 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 200µM dNTP, 10pM of 5' and 3' HGF/SF oligonucleotide primers, 2µl cDNA template and 1 U Taq polymerase (BRL). Amplification was performed for 45 cycles: 94°C (1min), 48°C (2min), 72°C (2min) in a Perkin Elmer Cetus DNA thermal cycler. The PCR products were separated on a 1.5% agarose gel and transferred to a Hybond N membrane (Amersham). An internal HGF/SF oligonucleotide (P3, 5'- 656 ACCTACAGGAAAACTACTG 675-3') (100ng) was end labeled using polynucleotide kinase. The membrane was prehybridized in 6 x SSC, 1% SDS, 5 x Denhardts solution and 200µg/ml salmon sperm DNA at 42°C for 1h and then hybridized in the same solution plus 50% formamide and 1 x 10^6 cpm/ml of labeled oligonucleotide probe at 42°C overnight. The membrane was washed with 2 x SSC and 0.1% SDS for 15' and then exposed to X-ray film.

Results

The *met*/HGF/SFR is expressed in epithelia in adult mouse tissues.

Transcripts for the *met*/HGF/SFR have been identified in a wide variety of adult tissues by Northern hybridization (Chan et al. 1988; Iyer et al., 1990). To elucidate the physiological roles of this receptor we determined the cell type specific expression of the *met*/HGF/SFR in adult mouse tissues by *in situ* hybridization. Tissues positive for expression of the *met*/HGF/SFR by Northern hybridization were chosen for analysis. These include tissues with high levels of expression such as liver and kidney and tissues with lower levels of expression such as stomach, uterus, ovary and skin. Frozen sections of adult tissues were hybridized with two independent *met*/HGF/SFR antisense or sense riboprobes [(pSC-1 and pSC-2) Yang & Park, 1993] subcloned from cDNA for the murine *met*/HGF/SF receptor (Iyer et al., 1990) plus a non related gene, Hox-5.1 (Featherstone et al., 1988). Riboprobes were labeled with nonradioactive digoxigenin UTP and specific hybrids were detected with an anti-digoxigenin antibody linked with alkaline phosphatase. *Met*/HGF/SFR specific hybrids, visualized as blue/black precipitates, were identified in all tissues examined predominantly in epithelia.

Liver

HGF/SF is a potent mitogen for hepatocytes in culture and a high affinity receptor for HGF/SF was identified in plasma membranes prepared from liver (Higuchi et al., 1991). We show that in resting liver expression of the *met*/HGF/SFR was localized specifically to hepatocytes (Fig. 1B, Table 1). Expression was not detectable in other cell types of the liver including endothelial cells, kupffer cells and bile ductal epithelial cells. Control sections either hybridized with sense riboprobes (Fig. 1A) or pre-treated with RNAse and hybridized with an antisense pSC-1 or pSC-2 *met*/HGF/SFR riboprobe (data not shown) showed no specific hybridization.

Kidney

In addition to liver, high levels of *met*/HGF/SFR were detected by Northern hybridization in kidney (Fig. 5A). By *in situ* hybridization we have localized expression of the *met*/HGF/SFR in kidney to epithelial cells that lined the proximal and distal convoluted tubules in the cortex (Fig. 2B &C) and in squamous epithelium lining the Bowman's capsule (Fig 2C). However, expression of the *met*/HGF/SFR was not detected in the epithelial cells lining the collecting tubules in the medulla (Fig. 2B & C), or in endothelial cells, mesangial cells and other mesenchymal cells of the kidney (Fig. 2B).

Lung

In lung, *met*/HGF/SFR expression was specifically localized to columnar epithelial cells that line the bronchi and bronchioles (Fig. 2E & F. Table 1). *Met*/HGF/SFR expression was also detected in epithelial cells that line the alveoli (Fig. 2E & F. Table 1). These cells appeared cuboidal and most probably represented type II pneumonocytes. This was not apparent in control sections of lung tissue either hybridized with sense *met*/HGF/SFR riboprobes (Fig. 2D) or sections pretreated with RNase and hybridized with antisense probes (data not shown). Smooth muscle cells, lymphocytes and endothelial cells lining the blood vessels are equivocal for *met*/HGF/SFR expression (Fig. 2E and F).

Oesophagus and Stomach

Strong *met*/HGF/SFR specific hybridization was localized to the stratified squamous epithelia of the oesophagus and the glandular epithelial cells that lined the gastric mucosa (Fig. 2K & L. Table. 1). Within the submucosa *met*/HGF/SFR expression was identified in the ganglion cells of postganglionic parasympathetic neurons of the *Meissner's* plexus. In addition some scattered cells in the submucosal mesenchyme were also positive for *met*/HGF/SFR expression, however, the nature of these cells could not be definitely identified (Fig. 2K).

Smooth muscle cells of the muscularis mucosae and propria showed no detectable levels of *met*/HGF/SFR expression.

Uterus and Ovary

By Northern hybridization low levels of *met*/HGF/SFR expression were detected in uterus, ovary and skin. Within the uterus the *met*/HGF/SFR expression is restricted to columnar epithelial cells that line the uterine lumen (Fig. 2H & I, Table 1) whereas the uterine glandular epithelial cells, stromal cells within the endometrium and myometrium show no specific *met*/HGF/SFR hybridization. In ovary we have localized *met*/HGF/SFR expression in the follicular granulosa cells of developing (Fig. 3C), mature (Fig. 3D) and ruptured follicles (Fig. 3E). However no *met*/HGF/SFR specific hybridization is detected in the epithelial follicular cells that envelop the oocyte. Similarly *met*/HGF/SFR is not detectably expressed in the stroma or surface epithelial cells of the ovary (Fig. 3B).

Skin

The epidermis of skin is a layer of stratified squamous epithelial cells. Although *met*/HGF/SFR expression has been demonstrated in keratinocyte cell lines in culture (Bottaro et al., 1991), only a few cells in this layer express *met*/HGF/SFR specific transcripts at levels detected by *in situ* hybridization (Fig. 4B & C). Within skin, *met*/HGF/SFR expression is predominantly localized to some cells forming the skin appendix structure, and on a closer examination these appear to localize to the glandular epithelial cells that line sweat glands (Fig. 4B, C & D). Whereas the connective tissue of the subcutaneous layer and epithelial cells surrounding the hair follicles show no specific hybridization, additional cells within the dermis also stained positive. Although we cannot unequivocally identify these cells, from hematoxylin and eosin (H & E) staining they most likely correspond to fibroblast like cells.

Expression of HGF/SF RNA in adult mouse tissues.

To investigate if HGF/SF is expressed in tissues where the *met*/HGF/SFR is expressed we compared *met*/HGF/SFR and HGF/SF expression by Northern Hybridization. We confirmed data previously obtained from rat tissues (Nakamura et al., 1986) that moderate levels of the 6kb transcript for HGF/SF is detected in adult mouse liver and that lower levels are detected in lung and kidney (Fig. 5B) To establish if HGF/SF is expressed in adult tissues which show no signal by Northern hybridization we used reverse transcription polymerase chain reaction amplification. First strand cDNA was synthesized and aliquots were subjected to PCR amplification using HGF/SF oligonucleotide primers derived from both the 5' and 3' portions of the HGF/SF transcript (P1-P2, and P3-P4 see materials and methods). The authenticity of the PCR product was confirmed by Southern transfer and hybridization with an internal HGF/SF specific oligonucleotide primer (P3) (Fig. 6). A PCR product of the expected size of 260bp for HGF/SF P1 and P2 primers was detected in cDNA prepared from adult liver, kidney, lung, stomach, uterus, intestine and low levels are detected in ovary but no HGF/SF specific product was detected in skin. All PCR reactions were confirmed using a second set of HGF/SF oligonucleotide primers (P4 and P5) which generate a 278bp specific HGF/SF PCR product. Oligonucleotide primers for β-actin and c-Myc were used in PCR reactions to control for cDNA quality and concentration (data not shown).

Discussion

The *met*/HGF/SFR receptor tyrosine kinase was originally identified as an activated oncogene and subsequently identified as the receptor for HGF/SF (Cooper et al., 1984; Park et al., 1987; Bottaro et al., 1991; Naldini et al., 1991b). HGF/SF is considered an important mitogen for liver regeneration and promotes the growth, motility and morphogenesis of epithelial and endothelial cells in culture. However, in addition to liver, from Northern hybridization analyses the *met*/HGF/SF receptor is expressed in many adult mouse tissues (Chan et al., 1988; Iyer et al., 1990). As a step towards understanding the biological function of the *met*/HGF/SFR in the adult animal we have analyzed the specific cell type expression of the *met*/HGF/SFR in adult mouse tissues by *in situ* hybridization.

We show that *met*/HGF/SFR expression is localized to hepatocytes in resting liver and is predominantly localized to tubular epithelia that line the proximal and distal convoluted tubules, Bowman's capsule of the kidney, as well as surface epithelia of stomach, oesophagus, lung and uterus. *Met*/HGF/SFR expression has been localized to the epithelia of various developing organs during embryogenesis (Sonnenberg et al., 1993) and we show here that this expression pattern is maintained in fully differentiated epithelia in these adult tissues. We also show that *met*/HGF/SFR is expressed in granulosa cells in developing and mature oocytes. Granulosa cells differentiate from epithelia of the sex cords, which is consistent with the general localization of *met*/HGF/SFR expression to epithelia.

In adult tissues, *met*/HGF/SFR expression had previously been localized to hepatocytes and endometrial epithelia by immunohistochemistry (Prat et al., 1991). Our results are in agreement with these and other studies that identified high affinity binding sites for HGF/SF in a variety of tissues including liver, kidney and lung (Tajima et al., 1992). *Met*/HGF/SFR immunoreactivity was also reported in breast ductal epithelia (Tsarfaty et al., 1992). Although we did not

examine this tissue, we show that the *met*/HGF/SF receptor is expressed in the epithelia that line sweat glands. Both mammary and sweat glands develop from the same precursor germinative basal epidermal cells in the dermis. Thus, *met*/HGF/SFR expression in both of these epithelia is consistent.

HGF/SF was originally purified from plasma, embryonic fibroblasts and smooth muscle cells in culture (Nakamura et al., 1986; Zarnegar et al., 1989; Rosen et al., 1989). By RT-PCR amplification we have demonstrated that with the exception of skin, HGF/SF is expressed in many tissue types *in vivo* , including intestine, liver, kidney, stomach, lung, uterus and ovary. However expression of HGF/SF in many of these tissues is low and can not readily be detected by Northern hybridization (Fig. 5B) or by *in situ* hybridization (data not shown). Although HGF/SF immunoreactivity had previously been localized to lung, kidney and liver epithelia (Defrances et al., 1992), the expression of the *met*/HGF/SFR receptor in these epithelia, suggests that the HGF/SF immunoreactivity may correspond to HGF/SF associated with its receptor.

During embryogenesis *met*/HGF/SFR expression is localized to many developing epithelia (Sonnenberg et al., 1993). However, in the adult animal, the majority of cell differentiation has ceased. Nevertheless, all epithelia are labile structures and are renewed continuously by means of mitotic activity. Repair of a damaged epithelium is in part dependent on regeneration through mitosis but is also partly a consequence of invasion by the nearby surviving epithelial cells (Cunha, 1985). HGF/SF stimulates epithelial cell mitogenesis, motility and morphogenesis by activating the kinase activity of the *met*/HGF/SFR (Weidner et al., 1993; Komada & Kitamura, 1993; Zhu et al., 1994). Since many mesenchymally derived cell lines in culture are known to synthesize HGF/SF and we have localized the *met*/HGF/SFR receptor to epithelia in many adult tissues, it seems likely that HGF/SF acts as a paracrine or endocrine mediator for mesenchymal-epithelial cell interactions in these tissues. Since we show that

HGF/SF RNA is also expressed in various organs (Zarnegar et al., 1990), the wide distribution of the *met*/HGF/SFR to epithelia, suggests that in addition to a role in liver regeneration, the *met*/HGF/SFR may play a role in organogenesis, repair and homeostatic tissue organization in many organs. The observation that increased levels of *met*/HGF/SFR mRNA are found in many carcinomas as well as in epithelial tumor cell lines in culture, suggests that its increased expression may also confer a growth advantage to neoplastic epithelial cells.

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Figure 1. Expression of the *met*/HGF/SFR in liver.

In situ analysis is performed using a digoxigenin-labeled cRNA probe on frozen sections of liver (10μ). Hybridization with a sense (A) or antisense (B) *met*/HGF/SFR probe. Specific hybrids are visualized as blue precipitates. The arrow indicates the positive staining in hepatocytes. Bar = 0.02 mm.

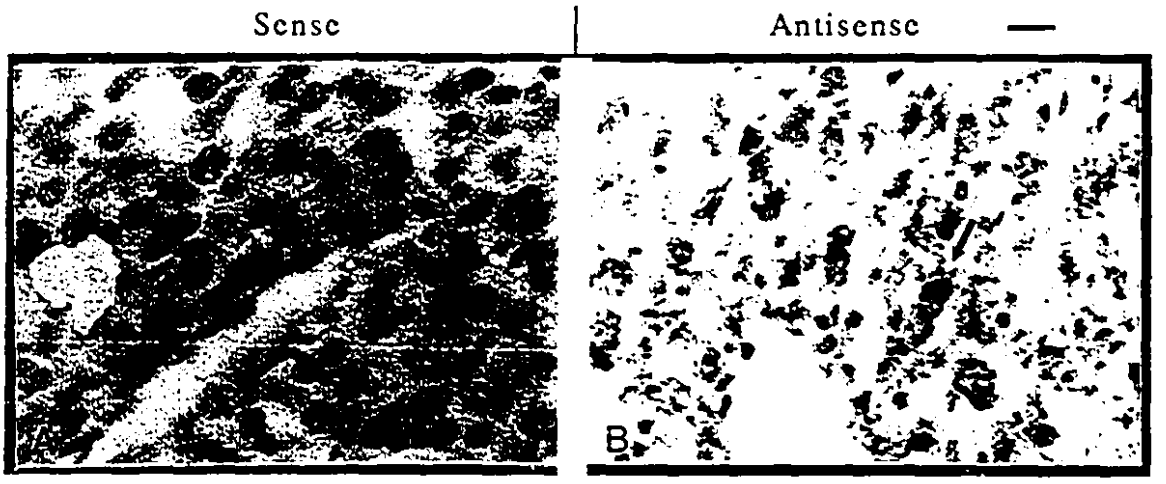
Sense
Antisense
B

Figure 2. The *met*/HGF/SFR is expressed in luminal epithelial cells.

Sections from Kidney (A, B & C), Lung (D, E & F), Uterus (G, H & I) and Stomach (J, K & L) were hybridized with sense (left panel) and antisense (middle and right panels) *met*/HGF/SFR riboprobes. Arrows indicate cells that show positive hybridization. C. columnar epithelial cells lining proximal convoluted tubules of the kidney; E. Type II pneumonocytes; F. columnar bronchial epithelia; I. columnar epithelial of uterine lumen; K. stratified squamous epithelia, oesophagus; L. glandular epithelial cells, stomach. Bar in A, B, J & K = 0.16 mm. Bar for D, E, G & H: 0.08 mm. Bar for high Magnification: 0.02 mm.

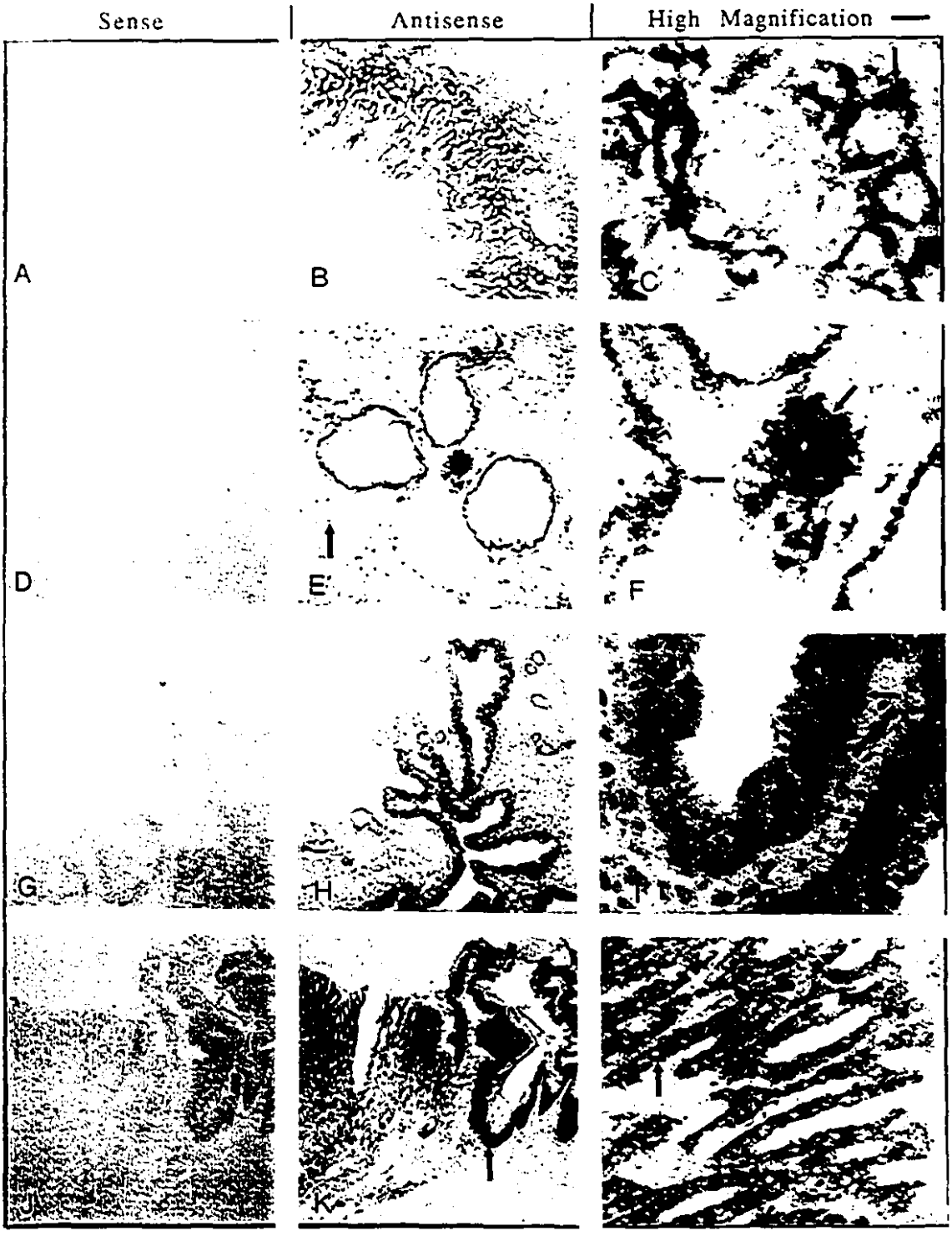
Sense
Antisense
High Magnification
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3. The *met*/HGF/SFR is expressed in granulosa cells in the ovary.

Frozen sections of ovary were hybridized with sense (A) and antisense (B, C, D & E) *met*/HGF/SFR probes. C and D: Mature follicle. E: Ruptured follicle. Granulosa cells are indicated (arrow). Bar for A & B: 0.08 mm; Bar for C, D & E: 0.02 mm.

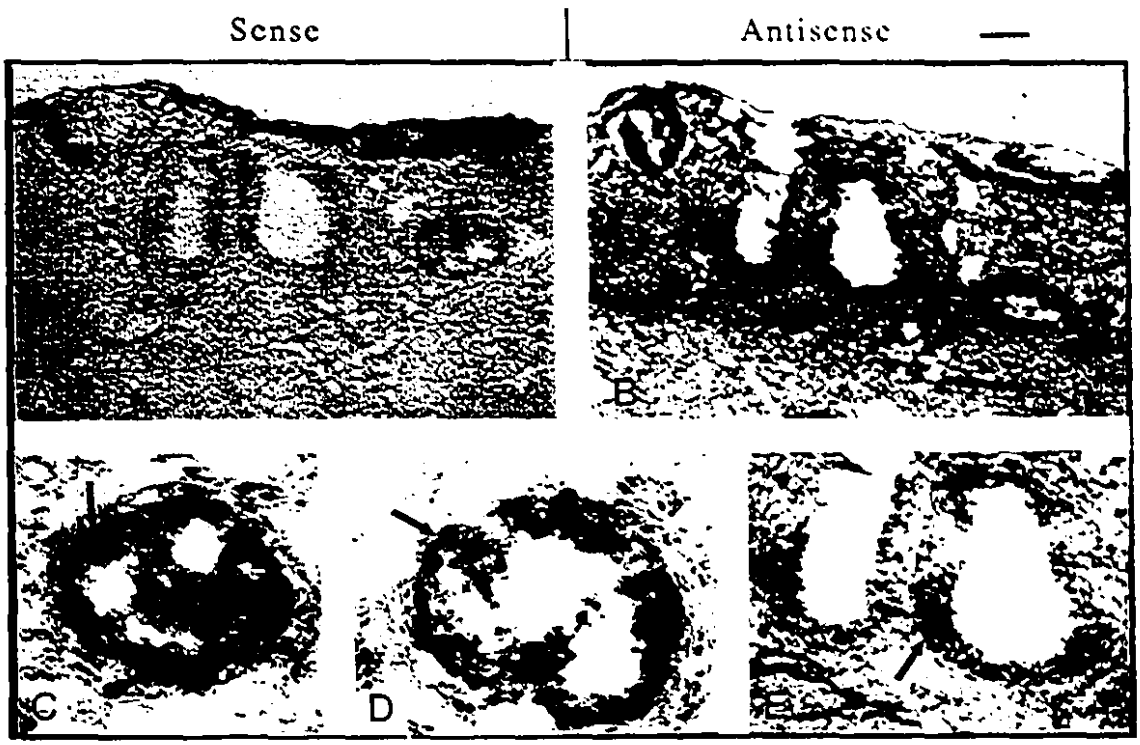
Sense
Antisense
B
C
D
E

Figure 4. The *met*/HGF/SFR is expressed in glandular epithelial cells in skin. Frozen section of skins were hybridized with sense (A) or antisense (B, C & D) *met*/HGF/SFR probes. Epithelial cells of sweat glands are indicated by the arrow and the fibroblast-like cells are indicated by the arrowhead. (E & F) H & E staining . Bar for A, B & E; 0.02 mm; Bar for C: 0.001; D & F: 0.0086 mm.

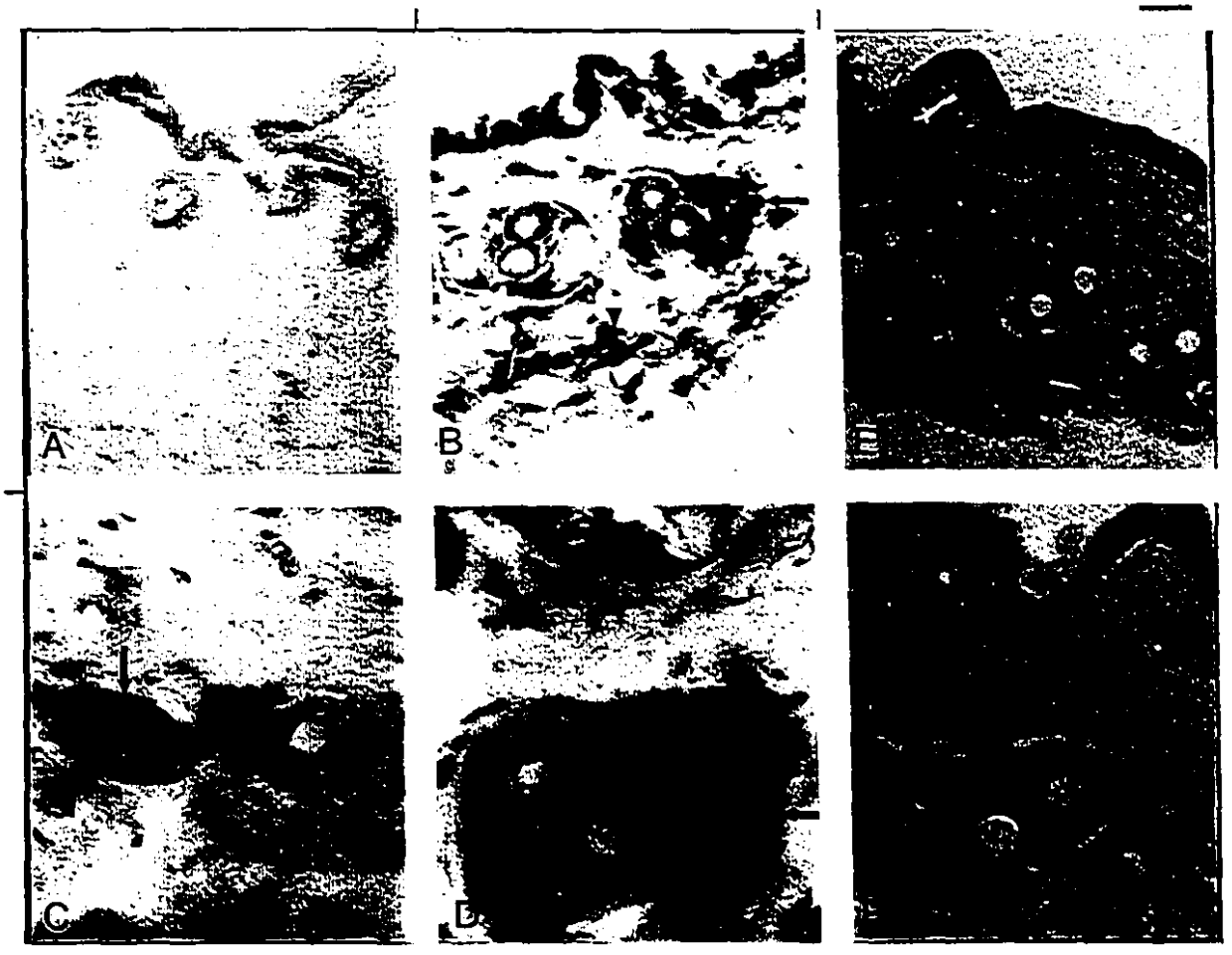
A
B
C
D

Figure 5. Northern hybridization analysis of the *met*/HGF/SFR and HGF/SF expression in adult mouse tissues.

40 µg of 1 X Poly A selected RNA was loaded in each lane . Hybridization with a 2.1 Kbp EcoR I 3' fragment of the mouse *met*/HGF/SFR cDNA , pcD (A), or with a 1.4 Kbp EcoR I 3' fragment of the rat HGF/SF cDNA, pRBC1 (Tashiro et al., 1990). (B). Control hybridization with a rat β-actin probe (C).

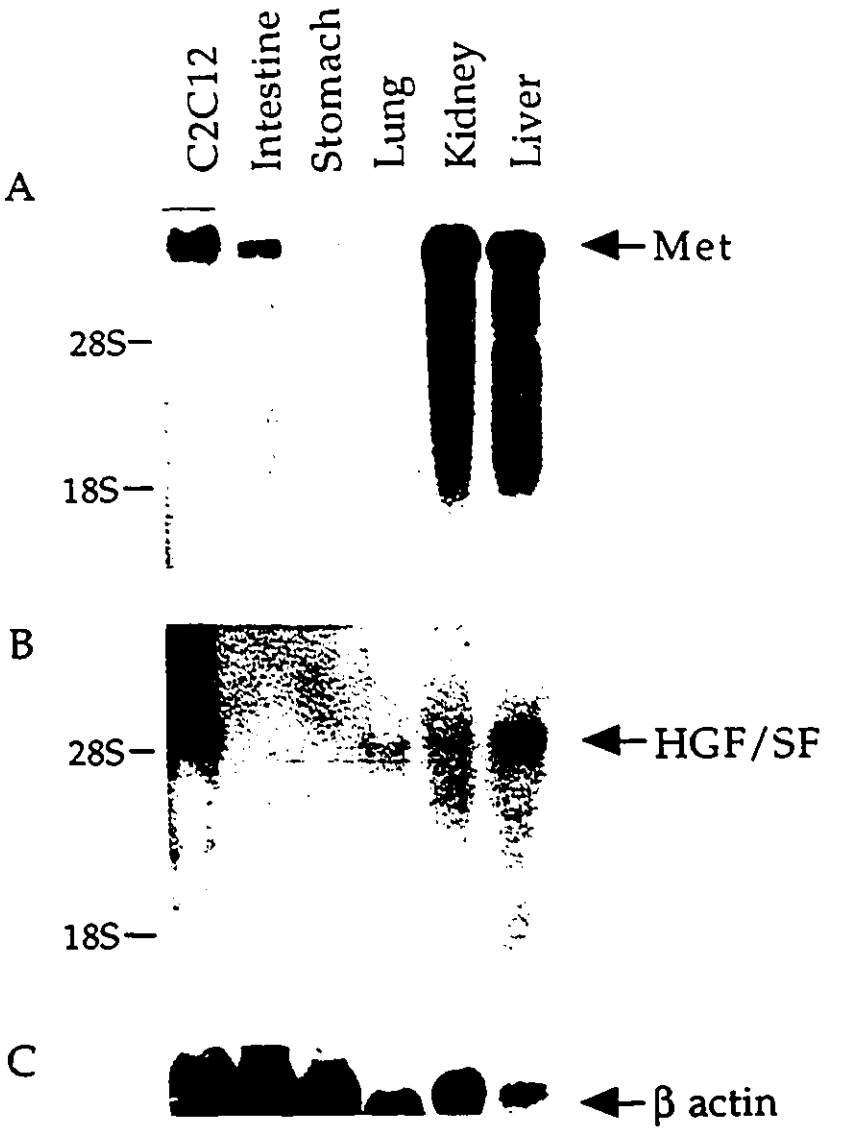
C2C12
Intestine
Stomach
Lung
Kidney
Liver
A
Met
28S
18S
B
HGF/SF
28S
18S
C
β actin

Figure 6. PCR amplification of HGF/SF.

First strand cDNA was synthesized from mRNA isolated from various mouse tissues with or without transcriptase (tissues lane labeled with -). The cDNA was amplified by PCR using HGF/SF oligonucleotide primers. PCR product was analyzed by gel electrophoresis, transferred to a Hybond N membrane, and hybridized with a radiolabeled internal HGF/SF oligonucleotide primer. The HGF/SF specific product of 260 bp is indicated. As control no cDNA template was used in the PCR amplification reaction.

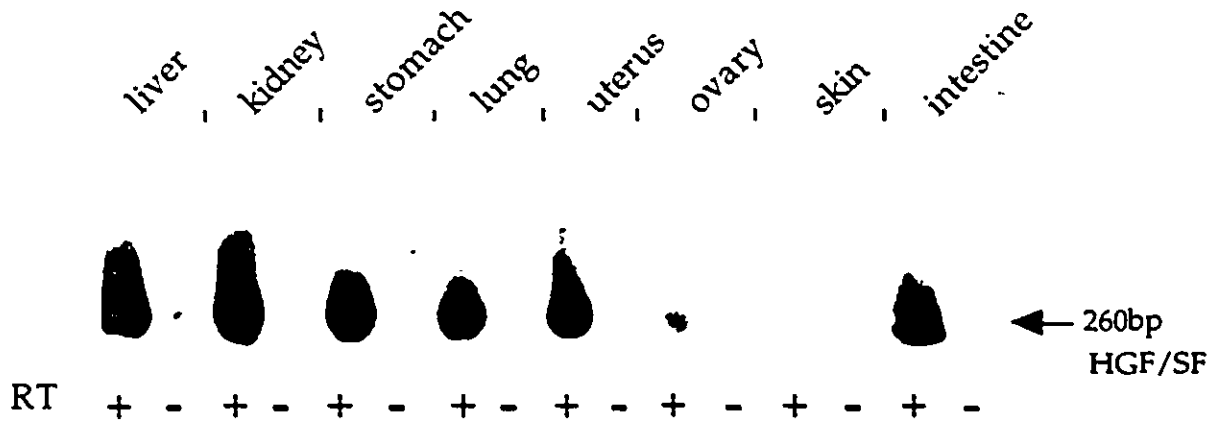
liver
kidney
stomach
lung
uterus
ovary
skin
intestine
260bp
HGF/SF
RT
+ - + - + - + - + - + - + - + -

Table 1 Expression of the *met*/HGF/SFR in adult mouse tissues

Tissues	*met*/HGF/SFR expression	HGF/SF expression
Liver	Hepatocytes	+
Kidney	Epithelial cells in proximal and distal convoluted tubules, and Bowman's capsule	+
Lung	Bronchial epithelial cells, Type II pneumonocytes	+
Stomach	Surface epithelial cells, Ganglion cells in submucosa	+
Intestine	Surface epithelial cells	+
Uterus	Luminal epithelium	+
Ovary	Granulosa cells in mature oocytes and ruptured follicles	+/-
Skin	Epithelial cells of sweat glands	-

Chapter III Expression of The *Met*/Hepatocyte Growth Factor/Scatter Factor Receptor And Its Ligand During Differentiation Of Murine P19 Embryonal Carcinoma Cells

Preface

Previous reports demonstrated that the *met*/HGF/SFR may play a role in cell proliferation, migration, tubulogenesis of many epithelial cells. The fact that the HGF/SF is predominantely mesenchymal derived and its receptor is expressed in epithelial supports a possible role of the *met*/HGF/SFR in the interaction of epithelial and mesenchymal cells which is an important cellular process during cell differentiation and embryonic development. In addition the *met*/HGF/SFR expression was detected in embryonic and extra-embryonic tissues by Northern blot hybridization, suggesting that the *met* receptor may have a function in development and cell differentiation. To investigate the expression of the *met*/HGF/SFR during cell differentiation, I chose to use the P19 embryonic carcinoma cells which are thought to be resemble the inner cell mass of the pre-implantation embryo. The P19 cells are pluripotent and can be induced to differentiate into neuronal and mesodermal derivatives. In this manuscript I describe that the *met*/HGF/SFR and its ligand are expressed at a low level in undifferentiated P19 cells, and that their expression is increased as the P19 cells differentiate into neuronal and mesodermal derivatives following treatment with RA and DMSO. The biological implications of such an observation are discussed.

Abstract

The *met* proto-oncogene is a member of the family of tyrosine kinase growth factor receptors and was recently identified as a receptor for hepatocyte growth factor and scatter factor (HGF/SF). From Northern hybridization studies the *met*/HGF/SF receptor (R) is expressed in many adult and embryonic mouse tissues. To identify which specific differentiated cell types express the *met*/HGF/SFR and to investigate the biological function of this receptor and its ligand during early murine development, we chose to study the expression of the *met*/HGF/SFR and HGF/SF during differentiation of the pluripotent P19 murine embryonal carcinoma cell line in culture. In this paper we demonstrate that *met*/HGF/SFR mRNA, protein and its ligand are expressed at a low level in undifferentiated P19 cells, and that their expression is increased as P19 cells are induced to differentiate into neuroectodermal derivatives following treatment with retinoic acid (RA) and into mesodermal derivatives following treatment with dimethyl sulphoxide (DMSO). From *in situ* hybridization analyses, only a subpopulation of differentiating P19 cells treated with RA or DMSO express high levels of the *met*/HGF/SFR RNA. In cultures treated with RA and cytosine arabinoside, both *met*/HGF/SFR mRNA and protein can be localized specifically to non-dividing neuronal cells. Expression of the *met*/HGF/SFR in undifferentiated P19 cells and differentiated derivatives suggests that stimulation of this signal transduction pathway may be an important event for the control of cell differentiation, proliferation or positioning during embryogenesis.

Introduction

The generation of many different cell types from a single pluripotent cell during embryogenesis is a highly regulated process in which peptide growth and differentiation factors have been shown to play essential roles (Adamson, 1987). This implies that stage and cell type specific expression of receptor molecules regulates many of the signaling processes required during embryonic development. A large number of these cell surface receptors belong to a family of receptor tyrosine kinases (RTK), which are characterized by an extracellular domain, a single hydrophobic transmembrane domain, and a tyrosine specific protein kinase activity in their cytoplasmic domain (reviewed in Yarden and Ullrich, 1988).

The human *met* gene was first isolated as an activated oncogene in an N-methyl-N'-nitronitrosoguanidine (MNNG) treated human osteogenic sarcoma cell line (HOS) by its ability to transform NIH3T3 mouse fibroblasts (Cooper et al., 1984). The structural and functional properties of the *met* proto-oncogene demonstrated that it is a member of the receptor tyrosine kinase gene family (Park et al., 1987) and was recently identified as a receptor for hepatocyte growth factor and scatter factor (HGF/SF) (Bottaro et al., 1991; Naldini et al., 1991b; Bhargava et al., 1992). The human and mouse *met*/HGF/SFR genes encode glycosylated, transmembrane proteins of 190 kDa, which consist of a 50 kDa α subunit exposed only on the cell surface, disulfide linked to a 140 kDa β subunit that spans the plasma membrane and contains the catalytic kinase domain (Gonzatti-Haces et al., 1988; Giordano et al., 1989a,b; Rodrigues et al., 1991). The unique structure of the p190 *met*/HGF/SFR product suggests that it is the prototype for a new class of receptor which is distinct from the known classes of receptors represented by the epidermal growth factor (EGF), platelet derived growth factor and the insulin receptors. Treatment of cells with HGF/SF results in autophosphorylation of the p190 *met*/HGF/SFR on tyrosine residues (Bottaro

et al., 1991; Naldini et al., 1991b). This event *in vitro* has been shown to activate the intrinsic kinase activity of the p190 *met*/HGF/SFR (Naldini et al., 1991a) and by analogy with other receptor tyrosine kinases is required for signal transduction (reviewed in Ullrich and Schlessinger, 1990).

HGF/SF is a mesenchymal cell-derived protein that is a multifunctional cytokine. HGF/SF is a mitogen for primary hepatocytes, renal tubule cells and several epithelial cell lines in culture (Zarneger et al., 1990, Tashiro et al., 1990; Kan et al., 1991; Nakamura, T. 1991), whereas for other epithelial cell lines HGF/SF induces cell dispersion and motility (Stoker and Perryman, 1985; Gherardi et al., 1989; Rosen et al., 1989). Cell dispersion and motility are required for normal embryogenesis and tissue remodeling. Several studies have demonstrated that the *met*/HGF/SFR is expressed in many adult and embryonic mouse tissues (Chan et al., 1988; Iyer et al., 1990), but little information with respect to its possible function has been gained from these studies. To identify which specific differentiated cell types express the *met*/HGF/SFR and to study the biological function of the *met*/HGF/SFR during early murine development we sought to study the expression of the *met*/HGF/SFR and its ligand during differentiation of pluripotent stem cells in culture.

The P19 mouse embryonic carcinoma (EC) cell line is an undifferentiated stem cell line derived from a teratocarcinoma (Rudnicki and McBurney, 1987) and is frequently used as a convenient *in vitro* model system to study early events involved in embryonic differentiation and development (Martin, 1980; Adamson, 1987). P19 cells in culture show little tendency to differentiate but can be induced to differentiate following treatment with retinoic acid (RA) or dimethyl sulphoxide (DMSO) (McBurney, 1982). Following exposure to RA, P19 cells differentiate into neurons, astrocytes and fibroblast-like cells whereas treatment with DMSO leads to differentiation into mesodermal derivatives, including skeletal and cardiac muscle, as well as extraembryonic endoderm (McBurney et

al., 1982; Edwards et al., 1983a,b; Jones-Villeneuve et al., 1983; Mummery et al., 1987). In this paper we demonstrate that the *met*/HGF/SFR and its ligand, are expressed at a low but detectable level in undifferentiated P19 cells and that both are increased during P19 cell differentiation. From *in situ* hybridization of P19 cell populations we have shown that elevated levels of *met*/HGF/SFR transcripts are present in only a subpopulation of differentiating P19 cells and in P19 cell cultures treated with RA plus cytosine arabinoside (CA), *met*/HGF/SFR RNA and protein can be localized specifically to neuronal cells.

Materials and methods

Cell culture conditions

P19 cells were cultured as described by Rudnicki and McBurney (1987). Cells were maintained in Dubeecco's minimum essential medium (DMEM) supplemented with 7.5% (v/v) bovine and 2.5% (v/v) foetal bovine serum (FCS) [Bochnek Co], at 37^{0}C in a 5% CO_2 atmosphere. To induce differentiation, P19 cells in exponential growth phase were trypsinised in Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS) containing 0.025% trypsin and 1mM EDTA and plated at a density of 10^5 cells/ml in bacterial grade petri dishes, in DMEM with or without 0.5µM RA or 1% DMSO. The medium containing drugs was replaced after 3 days. Cell aggregates from the bacterial grade petri dishes were plated onto tissue culture dishes on day 6 in DMEM, after which the medium was changed every 2 days. All-trans retinoic acid [Sigma chemical Co] was prepared as a stock solution of 10^{-2}M in 95% ethanol and stored no more than 2 weeks at 4^{0}C in the dark.

To produce a population consisting primarily (greater than 90%) of neurons, 4 day old RA cell aggregates were trypsinised and the resulting cell suspensions were plated onto tissue culture dishes in fresh medium without RA. Two days later the medium was replaced with fresh medium containing 5µg/ml cytosine arabinoside. This treatment kills the proliferating non-neuronal cells and a neuron-enriched population was harvested at various times thereafter.

RNA extraction and northern hybridization

RNA was prepared following the protocol of Chomczynski and Sacchi 1987. Northern transfers were performed as described in Park et al., 1986. For each sample 40µg of total RNA was electrophoresed in a 1.2% agarose, 6% formaldehyde gel and blotted onto Hybond N membrane. Filters were

prehybridized in 0.5M Na_3PO_4, 1mM EDTA, 1% BSA, 7% SDS and 200µg salmon sperm DNA at 60°C for 1-4 hour, then hybridized in 30% formamide, 0.2M Na_3PO_4, 1mM EDTA, 1% BSA, 7% SDS and 200µg salmon sperm DNA and 10^6cpm/ml of radiolabelled probe of 2.1 EcoRI *met*/HGF/SFR 3' cDNA fragment. Filters were washed once in 2 x SSC, 0.1% SDS at room temperature for 20 min, then twice for 30 min in 0.1 x SSC, 0.1% SDS 65°C.

Plasmids

As probes for hybridization studies an 0.8 kb PstI fragment of glucose phosphate isomerase (GPI) (Gurney et al., 1986; Faik et al., 1988) and an 0.5kb EcoRI fragment (p*met* SC1, Fig. 2) derived from the 5' portion of the murine *met*/HGF/SFR cDNA pcD (Iyer et al., 1990) were subcloned into the pbluescript KSII+ vector (Stratagene).

RNase protection assay

For each lane, 40µg of total RNA or tRNA were co-precipitated with *met*/HGF/SFR and GPI riboprobes (5 x 10^5cpm per sample) prepared by *in vitro* transcription (Melton et al., 1984). Precipitates were dissolved in 50µl hybridization mix (4:1 of deionized formamide and 5x stock solution containing 200mM PIPES, pH6.4, 2M NaCl, 5mM EDTA), denatured for 5 minutes at 85°C and hybridized for 8-12 hours at 45°C. Hybrids were digested with 50 units of RNase T1 (Boehringer Mannheim) and 1.5µg RNase A (Sigma) in 10mM TrisHCl, pH7.5, 300mM NaCl and 5mM EDTA for 1h at room temperature. The digestion was terminated by addition of 1µl 2% SDS and proteinase K (50µg) 15 min at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Samples were denatured at 85°C for 5 minutes and loaded onto 6% denaturing acrylamide/urea gel.

In situ hybridization

P19 cell aggregates treated with RA or DMSO as described above were plated onto slides coated with 0.1% gelatin and allowed to differentiate for 4 days. RA treated cells were cultured on slides and treated with 5µg/ml cytosine arabinoside as described above. Cells were incubated in 4% paraformaldehyde in d'PBS for 15 min at RT, then fixed with ethanol/glacial acetic acid (95:5, v/v) at -20°C for at least 5 min. Preparations were air dried briefly and frozen at -80°C. For hybridization, slides were thawed to RT, hydrated and treated with proteinase K (10µg/ml) in 0.1M Tris-HCl pH 7.5, 50mM EDTA, 2mM $CaCl_2$ at 37°C for 20 min followed by incubation in 0.1M Triethanolamine containing 0.25% acetic anhydride for 10 min. For control slides, RNA was digested by treatment with 2µg/ml of RNAse A and RNAse T1 in 0.1M Tris, pH 7.5, 50mM EDTA and 4mM NaCl at 37°C for 1h prior to the proteinase K treatment. Slides were washed in DEPC-water for 5 min and 0.2% glycine PBS for 10 min, then prehybridized in a buffer containing 50% deionized formamide, 5x SSC, 5x Denhart's solution, 250µg/ml tRNA at 45°C for at least 1h. Cells were dehydrated in alcohol prior to hybridization.

Antisense and sense riboprobes were generated to the 0.5kb p*met* SC1 fragment by *in vitro* transcription using digoxigenin-UTP following the manufacturer's instructions (Boehringer Mannheim, Munjaal, 1990). Probes were diluted in prehybridization mix at 5ng/µl, and 40µl of hybridization mix was applied to each coverslip. Slides were incubated in a humid chamber at 42°C over night, then washed twice with 2x SSC, once with 0.2x SSC and twice with 0.1x SSC at 45°C for 15 min each and blocked with 2% normal sheep serum and 0.3% triton X-100 in buffer1 (100mM Tris-HCl, pH7.5, 150mM NaCl) for 30 min. To detect *met*/HGF/SFR specific hybrids, slides were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (1 to 5,000 dilution in buffer 1) for 30 min, then washed twice (15 min each) with buffer 1 and then

rinsed in buffer 3 (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM $MgCl_2$). The hybrids bound to anti-digoxigenin antibody can be visualized by a color reaction containing 337.5µg/ml nitroblue tetrazolium salt (NBT), 175µg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.24mg/ml Levamisole in buffer 3 and color was allowed to develop for 12 hours in the dark. The reaction was terminated by incubation with 100mM Tris-HCl, pH 8.0, 1mM EDTA for 5 min. Slides were dehydrated, incubated in xylene, mounted with permount and stored at 4°C in the dark. Slides were viewed and photographed by a light microscope.

Immunofluorescence assay

Cells treated with RA and CA were grown on poly-L-lysine coated slides, then fixed with 3% paraformaldehyde for 5 min at RT. After rinsing three times with 1:1 PBS and alpha-MEM (PBS-MEM), cells were incubated with anti-*met* peptide antibody (1:150) in PBS-MEM for 1h at 37°C. For the control slides, the anti-*met* peptide antibody was first preabsorbed with 10µg competing antigenic peptide for 5 min prior to adding to the slides, or no antibody was used. The slides were then washed three times with PBS-MEM for 5 min each and incubated with biotinylated anti-rabbit IgG antibody (1:500) (Bio/CAN) for 1h at 37°C. Slides were then washed with PBS-MEM and incubated with fluoresecent (FITC)-labeled Avidin D (Dimension) (1:500) for 30 min at 37°C. For double labelling, cells were then fixed with fresh methanol and acetic acid (95:5 v/v) for 10 min at -20°C and air dried briefly. These cells were rehydrated in PBS for 15 min, and then incubated with 2H3 monoclonal antibody (anti-NF 165Kda, Developmental bank, NIH) (1:100) in PBS for 1h at 37°C. After washing three times with PBS for 5 min each, the slides were incubated with rhodamine (TRITC)- conjugated affinipure Goat anti-mouse IgG antibody (Bio/CAN) (1:500) for 1h at 37°C. Slides were washed three times with PBS and mounted

with aqueous mounting medium and viewed under the immunofluorescence microscope.

In vitro **immunecomplex kinase assay**

Cells were rinsed with cold PBS and lysed in RIPA buffer (50mM Tris, pH8.0, 0.1%SDS, 0.5% sodium deoxycholate, 1% NP40, 150mM NaCl, 2µg/ml aprotinin and leupeptin) for 30 minutes on ice, then centrifuged for 10 min at 10,000g to remove insoluble material. The supernatants were stored at -80^{0}C. The protein concentration in cell lysates was quantified by a Bradford assay and 2mg of proteins were incubated with 10µl normal rabbit serum for 1h at 4^{0}C. Non-specific immunecomplexes were precipitated with protein A sepharose and the resulting supernatants were then incubated with 1µl anti-*met*-peptide antibody (Iyer et al., 1990) at 4^{0}C for 1h. For control samples, 10µg competing antigenic peptide was added prior to the antiserum. Immunoprecipitates were collected on protein A sepharose, washed once with RIPA buffer plus 5mM EDTA and twice with kinase buffer (25mM Hepes, pH7.5 and 10mM $MnCl_2$). Kinase assays were performed in 50µl kinase buffer containing 10µCi of [γ-^{32}P]ATP (specific activity, 7000 Ci/mmol) for 10min at 4^{0}C. Immunoprecipitates were then washed once in RIPA buffer, and twice RIPA plus low (150mM NaCl) and high (500mM NaCl) salt, samples were resuspended in reducing Laemmli sample buffer, boiled for 5 min and run on an 8% SDS-polyacrylamide gel (PAGE).

Western blot analysis

Cell aggregates from day 10, or undifferentiated P19 cells, were rinsed with cold PBS and lysed with a buffer containing 1% NP40, 50mM Tris (pH 8.0), 2mM EDTA, 150mM NaCl, 2µg/ml aprotinin and leupeptin, as well as 1mM sodium orthovanadate for 30 minutes on ice, then centrifuged for 10 minutes at 10,000g. The supernatants were quantified using a Bradford assay and 5mg of

proteins were incubated with 10µl anti-*met* peptide antibody (Iyer et al., 1990) at 4°C for 1 hour. For the control sample 10µg of competing antigenic peptide was added prior to the antiserum. Immunoprecipitates were collected on protein A sepharose and washed three times with the lysis buffer, then separated on an 8% SDS-PAGE. The gel was electrotransferred to nitrocellulose membrane. After blocking the non-specific binding with 3% BSA in TBST buffer (10mM Tris pH 8.0, 150mM NaCl, 0.1% Tween-20, 2.5mM EDTA), the membrane was incubated with the anti-*met* peptide antibody (1:150) in TBST buffer for 1h at RT. The membrane was washed four times with TBST buffer for 5 min each, and then incubated with the anti-horseradish peroxidase (1:5000) for 1h at RT. After washing with TBST, immunocomplexes were detected using the ECL detection kit (Amersham).

PCR amplification

Total RNA (5µg), isolated at various stages during P19 cell differentiation, was resuspended in 16µl of DEPC-treated water, denatured in 8µl of 0.1M methyl mercury and neutralized in 6µl of 0.7M b-mercaptoethanol. First strand cDNA was synthesized using 1.25µg random hexamers (Pharmacia) as primers as described in Park et al., 1987. The reaction was terminated, and cDNA was phenol/chloroform extracted and resuspended in 100µl dH_2O. To amplify an HGF/SF specific product of 260bp, two primers P1 (5'-CCATGAATTTGACCTCTATG-3') and P2 (5'-ACTGAGGAA-TGTCACAGACT-3') were selected (Tashiro et al., 1990). The PCR reaction contains 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 200µM dNTP, 10pM of 5' HGF/SF oligonucleotide primer and 3' HGF/SF primer, 2µl cDNA template and 1U Taq polymerase (BRL). Amplification was performed by 45 cycles: 94°C (1min), 48°C (2min), 72°C (2min) in a Perkin Elmer Cetus DNA thermal cycler. The PCR products were separated on a 1.5% agarose gel and transferred to

Hybond N membrane (Amershan). An internal HGF/SF oligonucleotide (P3, 5'-ACCTACAGGAAAACTACTG-3') (100ng) was endlabelled with [γ^{32}P] ATP using polynucleotide kinase. The membrane was prehybridized in 6 x SSC, 1% SDS, 5 x Denharts and 200µg/ml salmon sperm DNA at 42^{0}C for 1h and then hybridized in the same solution plus 50% formamide and 1 x 10^6 cpm/ml of labelled oligonucleotide probe at 42^{0}C overnight. The membrane was washed with 2 x SSC and 0.1% SDS for 15' and then exposed to the X-ray film. To amplify the b-actin gene (Alonso et al., 1986), two primers P4 (5'-AGCAAGAGAGGTATC-CTGAC-3') and P5 (5'-AGGGAGAGCATAGCC-CTCGT-3') were used in the PCR reaction. An internal radiolabelled b-actin primer P6 (5'-AGATCTGGCACCACACCTTC-3') was used to confirm the specificity of the amplified product.

Results

Expression of the *met*/HGF/SFR during differentiation of murine P19 embryonal carcinoma cells.

To determine if the expression of the *met*/HGF/SFR is modulated when P19 cells are induced to differentiate into a variety of mesodermal and neuroectodermal cell types, we examined the expression of the *met*/HGF/SFR by hybridization of RNA isolated at various times following treatment of P19 cells with either RA or DMSO. As shown in Figure 1 a *met*/HGF/SFR transcript of 8kb (Chan et al., 1988; Iyer et al., 1990), is expressed at a low but detectable level in undifferentiated P19 cells and aggregates without drug treatment. In differentiating P19 cell cultures treated with either 1% DMSO or RA ($5X10^{-7}M$) for 6 days the expression of the *met*/HGF/SFR was elevated when compared with untreated P19 cells. The high level expression of the *met*/HGF/SFR was maintained until days 10-12 after initiation of the experiment, at which stage, cardiac muscle, skeletal muscle and extraembryonic endoderm derivatives are present in DMSO treated cultures, whereas neurons, astrocytes and fibroblasts are present in the RA treated cultures (McBurney et al., 1982; Edwards et al., 1983a, b; Jones-Villeneuve et al., 1982, 1983). The amount of RNA loaded in each lane in figure 1 is indicated from ethidium bromide staining (Fig. 1, lower panel) and hybridization with glucose phosphate isomerase (GPI) (Fig. 1, middle panel) (Gurney et al., 1986; Faik et al., 1988). From densitometric scanning following normalization for RNA levels, the *met*/HGF/SFR expression was increased approximately 5 fold in the differentiating cultures when compared with untreated P19 cell aggregates.

To quantitate the increase in the *met*/HGF/SFR expression, we examined the accumulation of *met*/HGF/SFR transcripts in P19 cell aggregates treated with RA and DMSO using a sensitive ribonuclease protection assay. Equal amounts of total RNA (40µg), isolated at various times following induction of P19 cell

differentiation with RA or DMSO, was annealed with a *met*/HGF/SFR and GPI antisense riboprobe to control for RNA levels. A *met*/HGF/SFR riboprobe of 230bp was synthesized from the p*met* SC1 subclone (Fig. 2, lower panel) and protects a *met*/HGF/SFR specific hybrid of 150bp (Fig. 2 upper panel). The ribonuclease protection assays confirm that the level of *met*/HGF/SFR RNA is increased in DMSO and RA treated P19 cells when compared with undifferentiated P19 cells or untreated P19 cell aggregates (Fig. 2, upper panel). Spectrodensitometry tracing of the RNase protection assay in figure 2, and normalization for RNA levels demonstrated that by 16h after transfer onto tissue culture dishes the level of *met*/HGF/SFR expression had increased approximately 10 fold in DMSO treated and 5 fold in RA-treated P19 cells when compared with undifferentiated P19 cells (Fig. 2, middle panel).

The *met*/HGF/SFR is expressed in only a subset of differentiating P19 cells.

Although these data demonstrate quantitatively that the *met*/HGF/SFR expression is increased as P19 cells differentiate, it does not allow a qualitative evaluation of the *met*/HGF/SFR expression in the various differentiated cell types present. To identify which cells expressed the *met*/HGF/SFR, RA and DMSO treated P19 cell aggregates were cultured on gelatin coated coverslips and the *met*/HGF/SFR expression in the differentiating cells was analyzed by *in situ* hybridization. Cultures of differentiating P19 cells, at 8 days (data not shown) or 10 days after initiation of the experiment, were hybridized with both a sense and antisense *met*/HGF/SFR riboprobe (pSC1 Fig. 2, lower panel) labeled with digoxigenin UTP (Fig. 3). Hybrids were detected by an enzyme-linked immunoassay using an antibody-conjugate (antidigoxigenin alkaline phosphatase conjugate, <Dig>AP) as described in materials and methods. A subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-

indolylphosphate (X-phosphate) and nitroblue-tetrazolium salt (NBT) produces an insoluble blue/purple precipitate, which visualizes hybrid molecules (Fig. 3).

Hybridization was localized specifically to a subset of cells in the DMSO (Fig. 3B) and RA (Fig. 3C) treated aggregates and no hybridization was observed to untreated control cell aggregates (Fig. 3D). Although from Northern hybridization and RNase protection studies of untreated P19 cell aggregates show that the *met*/HGF/SFR is expressed at a low level, this is not detected by *in situ* hybridization. This suggests that the positively hybridizing cells in the RA and DMSO treated cultures correlate with differentiation of P19 cells. Any non-specific hybridization to cells in the RA or DMSO treated cultures was controlled by both hybridizing a *met*/HGF/SFR antisense probe to aggregates pretreated with RNase (data not shown), or by hybridizing aggregates with a *met*/HGF/SFR sense probe (Fig. 3B-D, upper panel). The sense probe showed no hybridization to either undifferentiated or differentiated P19 cells, further supporting our proposal that the antisense probe specifically hybridizes to *met*/HGF/SFR RNA only in differentiated P19 EC cells. When viewed under higher magnification (Fig 3B & C) only a subset of cells in the differentiated P19 cell population appeared to express *met*/HGF/SFR transcripts.

The *met*/HGF/SFR is expressed in non-dividing neuronal cells.

As an approach to identify which differentiated P19 EC cells express the *met*/HGF/SFR, a population of differentiated P19 cells consisting of greater than 90% neurons was generated. Treatment of RA-induced cell aggregates with cytosine arabinoside (CA), kills any proliferating cells such as astrocytes, glial and fibroblasts and by day 8 after treatment only non-dividing neuronal cells survive (Rudnicki and McBurney, 1987). In these conditions, a population of neuronal like cells with long processes can be observed under the light microscope (Fig. 4). A *met*/HGF/SFR antisense riboprobe hybridized to greater

than 90% of the neuronal enriched culture (Fig. 3A, lower panel) and no hybridization was obtained with the sense probe (Fig. 3D) or to an RNase treated culture (data not shown). The high percentage of cells hybridizing with the *met*/HGF/SFR antisense probe suggests that the *met*/HGF/SFR is expressed in these differentiated non-dividing neuronal cells.

To determine whether the *met*/HGF/SFR is expressed in the non-dividing neuronal cells, P19 cells treated with RA and CA were subjected to indirect immunofluorescence analysis. Cells were co-immunostained with an anti-*met* peptide antibody (Iyer et al., 1990) and an anti-165 kDa neurofilament (NF) antibody (monoclonal antibody, 2H3, developmental bank, NIH). Neurofilaments which are the intermediate filaments specific for neurons, consist of three polypetide subunits, one of which is the p165 NF (Jorgensen et al., 1976). Neurofilaments are expressed in differentiated neuronal cells and are not expressed in glia, fibroblasts or astrocytes (Raju et al., 1981; Jones-Villeneuve et. al., 1983). Therefore this antibody allows us to identify specifically non-dividing neuronal cells. From phase contrast microscopy, the majority of these cells contain long branched processes that form an interconnected network and resemble neurons (Fig. 4A & B, left panel). With the anti-NF antibody and rhodamine counterstain, over 90% of cells show positive staining (Fig. 4A & B, right panel). As expected both cell processes and cell bodies are stained with the anti-NF antibody. Localization of the *met*/HGF/SFR immunoreactivity with a fluorescein (FITC) counterstain, shows that the *met*/HGF/SFR and NF are coexpressed in the same cells. However, in these cells the *met*/HGF/SFR shows more intense staining in the cell bodies (Fig. 4A, middle panel). Incubating cells with an anti-*met* peptide antibody in the presence of 10μg competing antigenic peptide (Fig. 4B, middle panel), or incubation with the secondary antibody alone (data not shown), failed to show positive staining. This demonstrates therefore that these non-dividing neuronal cells, as identified using the anti-NF antibody,

also express *met*/HGF/SFR proteins. However, we can not exclude that other cell types in the RA treated P19 cell culture may express the *met*/HGF/SFR.

A functional *met*/HGF/SFR product is expressed in differentiated P19 cells.

Although differentiated P19 cells have an elevated level of *met*/HGF/SFR RNA, we wanted to determine if functional *met*/HGF/SFR proteins are also present. Lysates were prepared from undifferentiated and differentiated P19 cells and the *met*/HGF/SFR protein product was immunoprecipitated from equal amounts of cell lysate using excess anti-*met* peptide antibody (Iyer et al., 1990). Due to the relatively low amount of *met*/HGF/SFR RNA detected by Northern hybridization and RNase protection analyses, we chose to use a sensitive immune complex kinase assay to visualize *met*/HGF/SFR proteins (Gonzatti-Haces et al., 1988). In the presence of Mn^{++} and ATP the immunoprecipitated p140 *met*/HGF/SFR β subunit catalyses autophosphorylation on tyrosine residues *in vitro* (Gonzatti-Haces et al., 1988). Thus using γ^{32}P-ATP as a phosphate donor, phosphorylation of the p140 *met*/HGF/SFR β subunit can be visualized following SDS PAGE and autoradiography. Analysis of phosphorylated proteins by SDS-PAGE under reducing conditions showed that the 140 kDa *met*/HGF/SFR β subunit was detected at a low level in undifferentiated P19 cells and untreated P19 cell aggregates, and that immunoprecipitation of the p140 *met*/HGF/SFR β subunit is competed in the presence of antigenic peptide (Fig. 5). In comparison to the undifferentiated P19 EC cells and untreated P19 cell aggregates, increased levels of phosphorylated p140 *met*/HGF/SFR are present in the 8-12 day DMSO and RA treated aggregates (Fig 5).

To determine if the high levels of phosphorylated p140 *met*/HGF/SFR in day 10-12 RA treated cultures (Fig. 5) correlate with the appearance of neuronal cells, or are due to other cell types such as astrocytes, the levels of p140

met/HGF/SFR in RA treated P19 cell aggregates were compared with levels found in aggregates treated with RA and CA that contain only neuronal cells (Fig. 4A). In the RA plus CA treated cultures, the levels of phosphorylated p140 *met*/HGF/SFR remain high (Fig. 6). Thus neuronal cells express the p140 *met*/HGF/SFR β subunit, but we can not exclude the possibly that other differentiated cell types in the RA treated culture express the p140 *met*/HGF/SFR β subunit. Although this approach does not allow one to accurately quantify the amount of the *met*/HGF/SFR protein product, it demonstrates that the p140 *met*/HGF/SFR β subunit is synthesized and is functional, and that the level of the p140 *met*/HGF/SFR protein or kinase activity is increased in differentiating P19 cell cultures (Fig. 6).

To investigate if the level of the p140 *met*/HGF/SFR β subunit expression is increased during P19 cell differentiation, cell lysates were prepared from differentiated and undifferentiated P19 cells, and subjected to western immunoblot analysis. Protein concentration was determined using a Bradford assay and equal amounts of protein from each lysate were immunoprecipitated with an excess amount of anti-*met* peptide antibody (Iyer et al., 1990). Proteins were separated under reducing conditions on an 8% SDS-PAGE, transferred to a Nitrocellulose membrane, and blotted with the anti-*met* peptide antibody. These analyses confirm that the p140 *met*/HGF/SFR β subunit is expressed at a low level in undifferentiated and untreated P19 cell aggregates, but is increased in RA and DMSO treated P19 cells (Fig. 7), in addition to the neuronal enriched cell populations (RA+CA in Fig. 7).

Expression of HGF/SF during P19 cell differentiation.

The high level of the p140 *met*/HGF/SFR kinase activity in differentiating cultures of P19 cells, may not only correlate with increased expression of the p140 *met*/HGF/SFR but may also reflect stimulation of the *met*/HGF/SFR

kinase by its ligand HGF/SF. To examine if HGF/SF is expressed during P19 cell differentiation, a sensitive PCR amplification technique was used. Total RNA was isolated at various times during P19 cell differentiation and first strand cDNA was synthesized using reverse transcriptase. The resultant cDNA was amplified by the polymerase chain reaction (PCR) technique using HGF/SF oligonuleotide primers (P1 & P2) localized within the 5' portion of the HGF/SF transcript (Tashiro et al., 1990) (see materials and methods). To confirm that the amplified products correspond to HGF/SF, a southern blot of PCR products, separated by gel electrophoresis, was hybridized with an internal radiolabelled HGF/SF primer (P3) (Fig. 8A). As controls for the presence of contaminating DNA in the RNA preparations, RNA that had not been reverse transcribed was used as a template in PCR reactions (Fig. 8A, RT-). Since a PCR approach is only semi-quantitative, to allow us to quantify the relative levels of HGF/SF in these cDNA samples, each cDNA was also amplified using oligonucleotides specific for β-actin (P4 & P5) (materials and methods) (Schuuring et al., 1989; de Groot et al., 1990). The specificity and amount of β-actin product was confirmed by hybridizing with an internal β-actin primer (P6) (Fig. 8B). When compared with the β-actin RT-PCR, a low level of the expected HGF/SF product (260bp) is detected in cDNA prepared from undifferentiated P19 cells and this level increases when P19 cells are aggregated either in the absence or presence of RA or DMSO (Fig. 8A). This result was confirmed using a distinct set of HGF/SF specific oligonucleotide primers (data not shown). Although we see an increase in HGF/SF expression levels following aggregation, this is still not sufficient to detect by *in situ* hybridization. At the moment therefore, we are unable to identify which differentiated P19 cells synthesize HGF/SF.

Discussion

During embryogenesis the process of cell differentiation and proliferation is controlled by molecules that transmit signals within and between cells (Adamson, 1987). The demonstration that several developmental mutants map to genes encoding receptor tyrosine kinases has implicated these enzymes in this control process (Basler and Hafen, 1988; Chabot et al., 1988; Geissler et al., 1988). To study the role of the *met*/HGF/SFR in early mouse development we have characterized its expression in P19 EC cells induced to differentiate in culture. The data presented here show that the *met*/HGF/SFR and its ligand are expressed at low levels in undifferentiated P19 cells and that their expression is increased as P19 cells differentiate. An increase in the *met*/HGF/SFR RNA is detected when P19 cells are induced to differentiate into neuroectodermal derivatives following exposure to RA and into mesodermal derivatives following exposure to DMSO, whereas, an increase in HGF/SF RNA is observed following aggregation of P19 cells. From RNase protection analysis expression of the *met*/HGF/SFR is increased approximately 5-10 fold in differentiated P19 cells following treatment with RA or DMSO for 6 days. At the level of sensitivity of *in situ* hybridization, this increase in *met*/HGF/SFR expression was localized specifically to the differentiating cells and not to undifferentiated P19 EC cells (Fig 3). Since approximately 25-30% of the cells in the RA or DMSO induced P19 EC cell aggregates expressed detectable levels of the *met*/HGF/SFR by *in situ* hybridization, the relative level of expression of the *met*/HGF/SFR in these cells, as judged from RNase protection analysis, may be 15-30 fold higher than the level in undifferentiated P19 EC cells.

In the presence of 1% DMSO P19 cells differentiate into cardiac and skeletal muscle (Edwards et al., 1983b; Rudnicki and McBurney, 1987). Some extraembryonic endoderm like and various unknown cell types are also present but no astrocytes or neurons have been detected in 1% DMSO treated cell

cultures (McBurney et al., 1982; Edwards et al., 1983b). A previous study demonstrated that the *met*/HGF/SFR was expressed in yolk sac and amnion of mouse (Chan et al., 1988), suggesting that the *met*/HGF/SFR may be expressed in the extraembryonic cells in DMSO treated P19 cell aggregates. In addition, we have shown that a skeletal muscle precursor cell line, C2C12, expresses high levels of the *met*/HGF/SFR in both myoblasts and in differentiated myotubes (X-M Yang unpublished) and this may suggest a function for the *met*/HGF/SFR in muscle derivatives.

Hybridization *in situ* and indirect immunofluorescence staining of neuronal enriched P19 cell aggregates, revealed that *met*/HGF/SFR mRNA and protein is expressed in differentiated non-dividing neuronal cells (Fig. 3A and Fig. 4). Because these neuronal cells lack markers of mature neuronal cells, they appear to resemble embryonic rather than adult neuronal cells (Jones-Villeneuve et al., 1982, 1983). From Northern hybridization analysis a low level of expression of the *met*/HGF/SFR was detected in RNA prepared from both adult and embryonic mouse brains (X-M Yang unpublished results; Chan et al., 1988; Iyer et al., 1990), thus the *met*/HGF/SFR may be expressed in some neuronal derived cells *in vivo*. We have shown that the levels of HGF/SF increase following aggregation of P19 cells in the absence of RA or DMSO. When P19 cells are aggregated *in vitro* in normal culture medium, most cells retain the EC form, however, a small proportion differentiate into extra-embryonic endoderm like cells (Jones-Villeneure et al., 1982) suggesting that these cells may be a source for HGF/SF synthesis. However, other cell types in RA, DMSO and RA+CA treated P19 cell aggregates may also express HGF/SF. Since the level of HGF/SF expression is below the sensitivity for *in situ* hybridization, we are unable at this stage to identify which cells in these cultures express HGF/SF. Interestingly from immnohistochemical studies HGF/SF has been localized to large neurons of the brain (Zarneger et al., 1990). Furthermore, application of HGF/SF to early chick

embryos induced a secondary primitive streak (Stern et al., 1990). Thus the *met*/HGF/SF RTK signal transduction pathway may has a specific function in neuronal cell types.

We have demonstrated that in addition to RNA, the p140 *met*/HGF/SFR β subunit is also expressed in both undifferentiated and differentiated P19 EC cells. From *in vitro* kinase assays the p140 *met*/HGF/SFR has autophosphorylation activity and is thus expressed in a biologically functional form. In differentiated RA and DMSO treated P19 cultures, the increased level of the p140 *met*/HGF/SFR autophosphorylation is concordant with the increase in *met*/HGF/SFR protein levels. However we can not exclude that the increase in p140 *met*/HGF/SFR *in vitro* kinase activity in RA treated cultures may also result from partial stimulation of the *met*/HGF/SFR kinase by HGF/SF produced during differentiation of P19 EC cells. Although HGF/SF was initially considered to be an important factor in liver regeneration, it has now been shown to act as a mitogen for melanocytes and for several epithelial cells (Zarneger et al., 1990, Tashiro et al., 1990; Kan et al., 1991; Nakamura, T. 1991), and as a factor that stimulates the dissociation of tight junctions and movement of some epithelial cells in culture (Stoker et al., 1987; Gherardi et al., 1989; Weidner et al., 1991). Expression of HGF/SF has been localized primarily to fibroblasts in culture and to tissues of mesenchymal origin. From its sites of synthesis and target cells this implies that HGF/SF may play an important role as a paracrine mediator for cell proliferation and/or migration during embryogenesis.

During development the mesoderm delivers signal(s) capable of inducing the differentiation of neuroectoderm. This process is essentially mimicked during P19 EC cell aggregation where neuronal differentiation of P19 cells is dependent on RA. Neuronal differentiation of P19 EC cells can also be induced following stimulation of an ectopically expressed epidermal growth factor (EGF) receptor tyrosine kinase with EGF (den Hertog et al., 1991). This suggests that

the exogenously expressed EGFR substitutes for a RTK normally expressed in undifferentiated P19 cells. Although the expression of several members of the kinase family have been identified in undifferentiated P19 cells (Rees et al., 1979; Mummery et al., 1985; Sejersen et al., 1985; Boulter and Wagner, 1988; den Hertog et al., 1991) to our knowledge the *met*/HGF/SFR is the only receptor tyrosine kinase shown to be expressed in undifferentiated P19 cells. P19 EC cells resemble the inner cell mass of the pre-implantation embryo, thus, the *met*/HGF/SFR may be expressed prior to organogenesis *in vivo*, at a development stage as early as day 7 in mouse. Expression of the *met*/HGF/SFR and HGF/SF in undifferentiated P19 cells and differentiated derivatives indicates that activation of this signaling pathway may be an important event in cell differentiation, positioning or maintenance during embryogenesis. With the availability of purified HGF/SF, P19 cells are an important experimental system with which to investigate the possible role of HGF/SF/*met* stimulation on cell differentiation and proliferation.

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Figure 1. Northern blot hybridization analysis of the *met*/HGF/SFR expression during P19 cell differentiation.

RNA was isolated from untreated (-), RA and DMSO-treated P19 cell aggregates at 1-7 days after plating onto tissue culture dishes (day 7-14 in culture). Hybridization with a 2.1 EcoRI 3' fragment of the mouse *met*/HGF/SFR cDNA, pcD (upper panel). Control hybridization with an 0.8 Kb Pst I fragment of glucose-6-phosphate isomerase (GPI) cDNA (middle panel). Ethidium Bromide staining of RNA (lower panel).

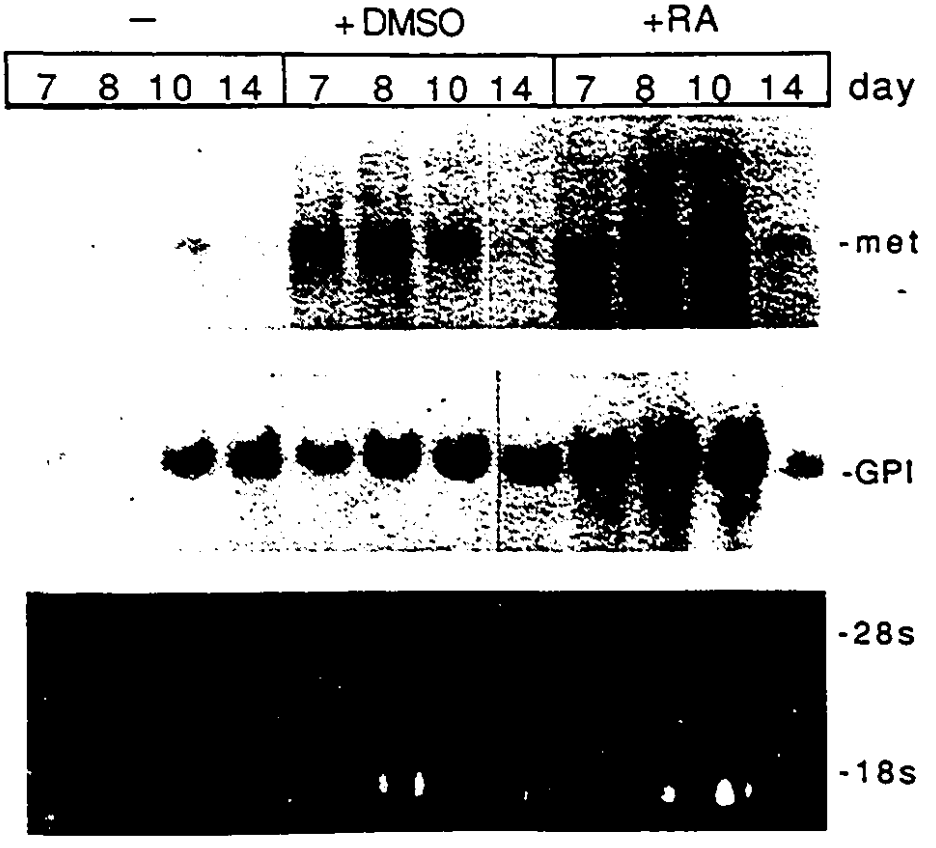

–
+DMSO
+RA
7 8 10 14 7 8 10 14 7 8 10 14
day
-met
-GPI
-28s
-18s

Figure 2. Quantitation of *met*/HGF/SFR RNA by RNase protection.

A ^{32}P-continuously labeled antisense riboprobe was hybridized to 40μg of total RNA from undifferentiated P19 cells (P19), untreated, DMSO, and RA-treated P19 cell aggregates at various times after plating onto the tissue culture dish. The protected fragment of 150bp corresponds to the predicted *met*/HGF/SFR transcript. ϕX 174 DNA digested with Hae III provides molecular weight markers (upper panel). Histogram of *met*/HGF/SFR RNA level determined by densitometric tracing and normalization to a GPI control riboprobe (middle panel). Schematic of the location of the probe, pSC1 in the murine *met* /HGF/SFR cDNA and the expected protected hybrid (lower panel).

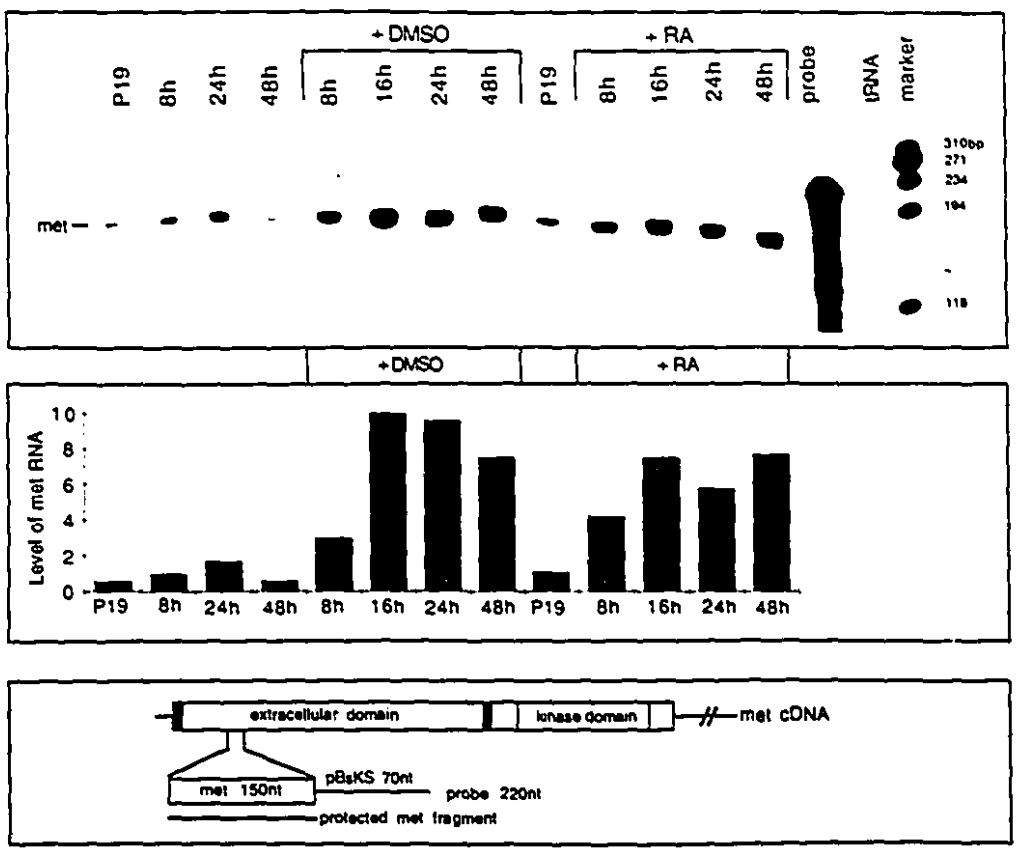

+ DMSO
+ RA
P19
8h
24h
48h
8h
16h
24h
48h
P19
8h
16h
24h
48h
probe
tRNA
marker
310bp
271
234
194
118
met
+ DMSO
+ RA
Level of met RNA
10
8
6
4
2
0
P19
8h
24h
48h
8h
16h
24h
48h
P19
8h
16h
24h
48h
extracellular domain
kinase domain
met cDNA
met 150nt
pBsKS 70nt
probe 220nt
protected met fragment

Figure 3. *In Situ* hybridization of the *met*/HGF/SFR expression in differentiated P19 cells.

Analysis is performed using a digoxigenin labeled riboprobe on P19 cell aggregates 4 days after plating on the tissue culture plates (day 10 in culture). Specific hybrids are visualized as blue/purple precipitates. The upper panels are control hybridizations with a sense *met*/HGF/SFR riboprobe. In the lower panels, hybridization is with an antisense *met*/HGF/SFR riboprobe. (A) Cytosine arabinoside and RA treated p19 cell aggregates. (B) RA-treated, (C) DMSO-treated, (D) Untreated cell aggregates. Bar in sense, antisense panel; A=0.15mm; B, C &D=1mm, Bar in high magnification panel; A=0.03mm; B, C & D= 0.08mm.

Figure 4. Immunofluorescence analysis.

Cells treated with RA and CA from Day 8 were co-incubated with anti-NF monoclonal antibody (2H3) (A & B, right panel) and anti-*met* peptide antibody in the absence (A, middle panel) or presence (B, middle panel) of competing antigenic peptide. The left panels in A and B are phase contrast pictures of the cells. Bar =0.01mm.

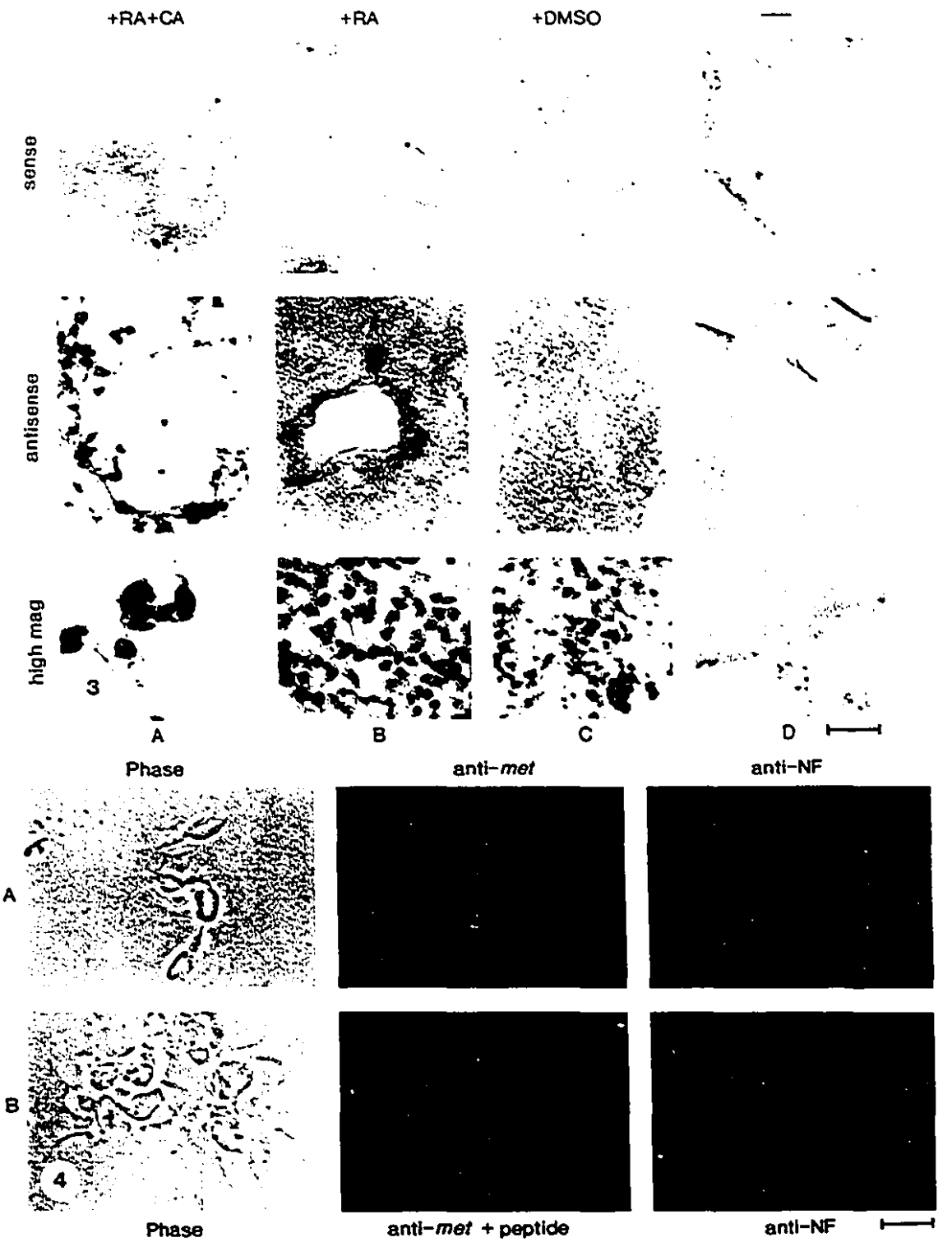

+RA+CA
+RA
+DMSO
—
sense
antisense
high mag
3
A
B
C
D
Phase
anti-*met*
anti-NF
A
B
4
Phase
anti-*met* + peptide
anti-NF

Figure 5. *In Vitro* immune complex protein kinase assay.

Cell lysates from undifferentiated P19 cells (P19), DMSO, RA-treated and untreated (-) cell aggregates at 1-6 days after plating onto tissue culture dishes (day 7-12 in culture). The P19 cell lysate was incubated with anti-*met* peptide antibody in the presence (+) or absence (-) of competing antigenic peptide. The resulting immunoprecipitates were subjected to an *in vitro* kinase reaction and proteins separated by an 6% SDS-PAGE as described in materials and methods. The phosphorylated p140 *met*/HGF/SFR product is indicated. High molecular weight markers (BRL) are shown. Myosin=205kDa, b-galactosidase=116.5kDa, bovine serum albumin=77kDa.

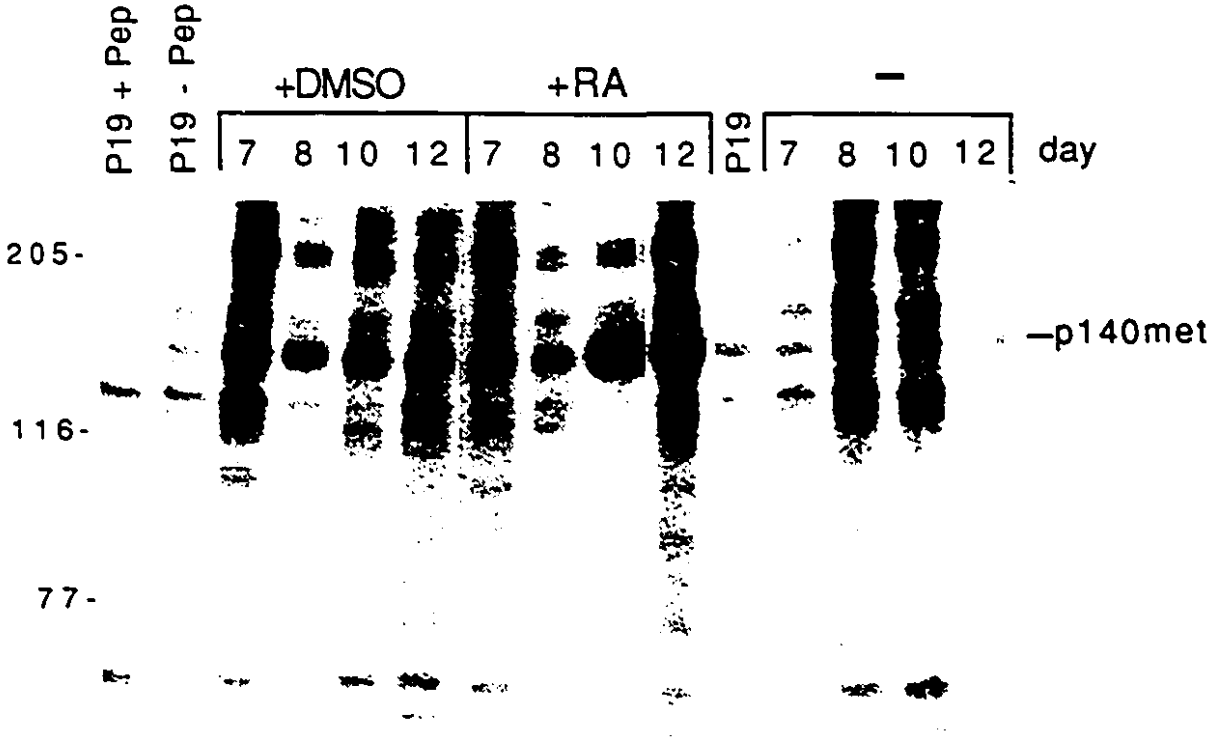

P19 + Pep
P19 - Pep
+DMSO
+RA
–
7 8 10 12 7 8 10 12 P19 7 8 10 12
day
205-
116-
77-
—p140met

Figure 6. *In vitro* immunecomplex kinase assay.

Cell lysates were isolated from day 8 or day 10 cell aggregates of untreated, RA-treated, and the RA and CA-treated P19 cells. Cell lysates were incubated with anti-*met* peptide antiserum in the presence (+) or absence of competing peptide (-). Immunoprecipitated proteins were subjected to an immune complex kinase assay and separated by an 8% SDS-PAGE. The phosphorylated p140 *met*/HGF/SFR protein product and molecular weight markers (as Fig 5) are indicated.


CA
RA
Day
8 8 8 8 10 8 10
-205KDa
p140met
-116
-77
peptide

Figure 7. Western immunoblot analysis.

Cell lysates from undifferentiated P19 cells (-), untreated (DMEM), DMSO, RA-treated aggregates, as well as RA +CA treated neuronal cells from day 10, were immunoprecipitated with anti-*met* peptide antibody with (+) or without (-) 10μg of competing antigenic peptide. Immunoprecipitated proteins were separated on an 8% SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with the anti-*met* peptide antibody. The p140 *met*/HGF/SFR β subunit and the molecular weight markers (as Fig. 5) are indicated.

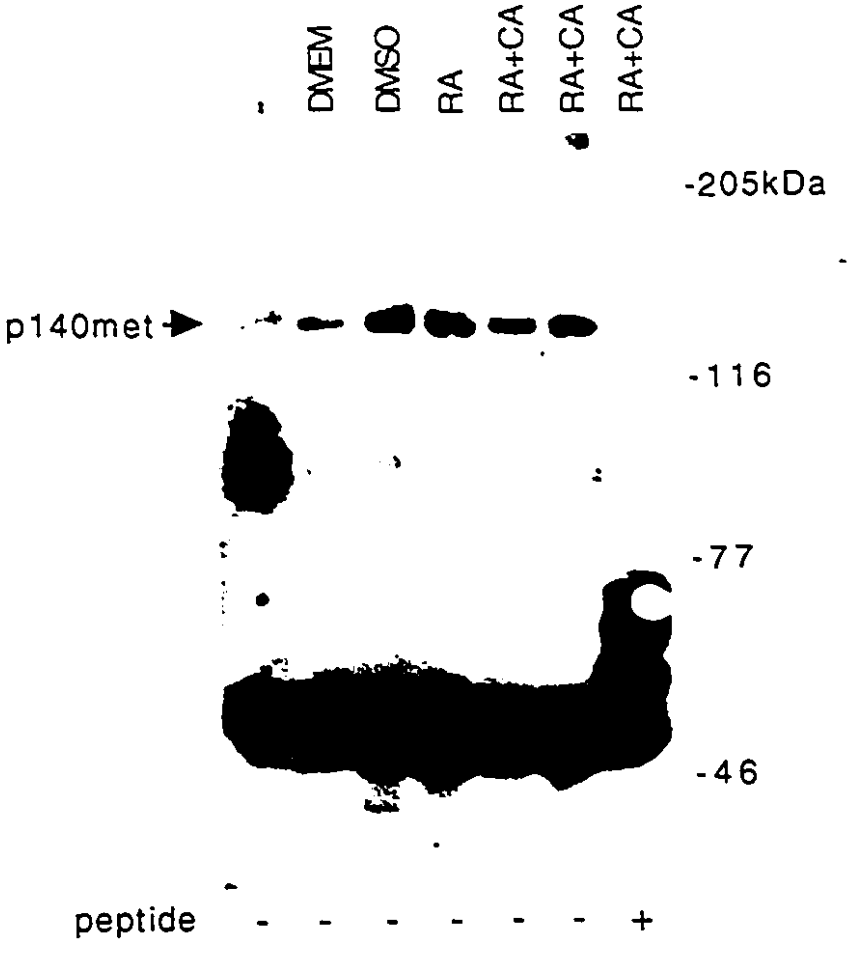
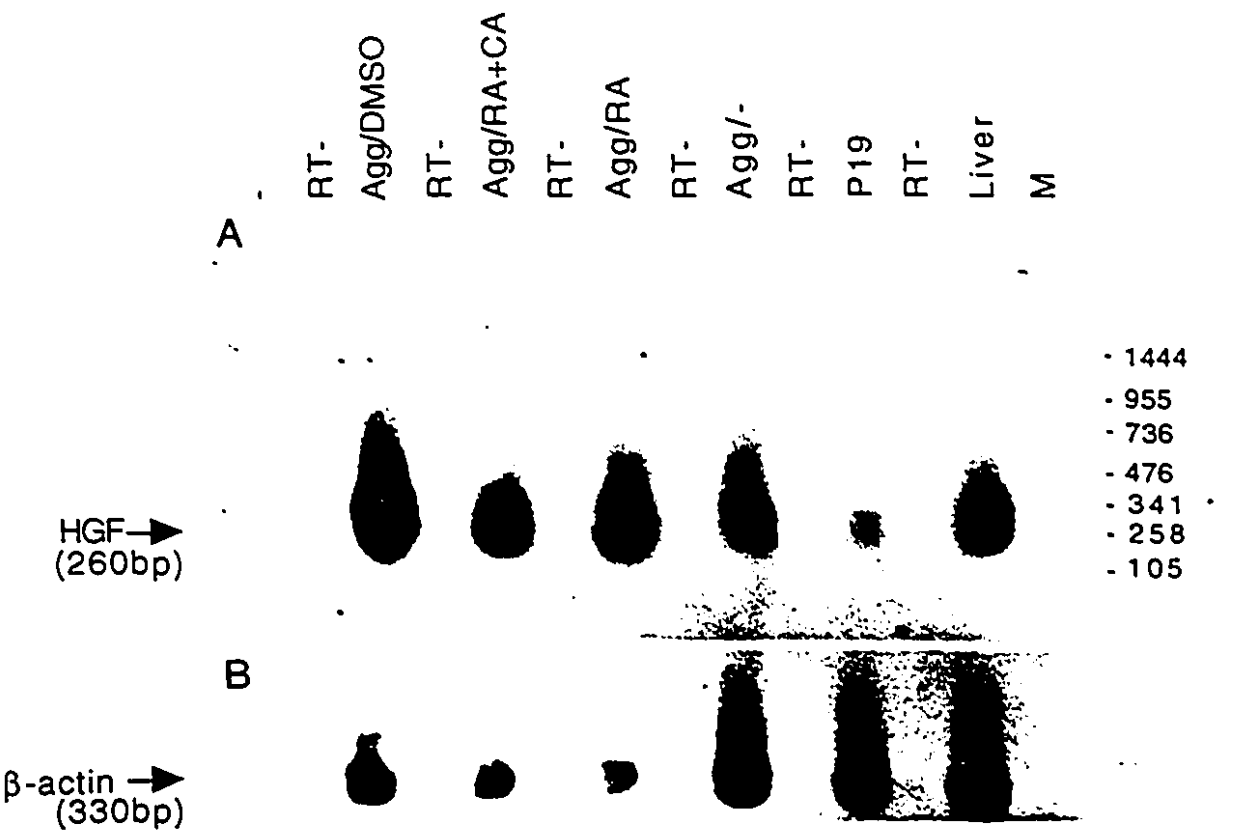

DMEM
DMSO
RA
RA+CA
RA+CA
RA+CA
-205kDa
p140met
-116
-77
-46
peptide - - - - - - +

Figure 8. PCR amplification of HGF/SF.

First strand cDNA was synthesized from mRNA isolated from liver, undifferentiated P19 cells (P19), as well as untreated P19 cell aggregates (Agg/-), RA-treated, DMSO-treated and RA plus CA treated day 10, P19 cell aggregates, with or without reverse transcriptase (RT-). The cDNA was amplified by PCR using HGF/SF oligonucleotide primers (P1 & P2, A) or b-actin oligonucleotide primers (P4 & P5, B). PCR products were analyzed by gel electrophoresis, transferred to Hybond N membrane, and hybridized with an internal radiolabelled HGF/SF oligonucleotide primer (P3 in A) or b-actin primer (P6 in B). The HGF/SF specific product of 260bp (A) and b-actin specific product of 320bp (B) are indicated. The last lane (-) represents a control where no cDNA template was used in the PCR amplification reaction. PUC 19 TaqI/Sau3A1 DNA size markers (Stratagene) are labeled (M).


RT-
Agg/DMSO
RT-
Agg/RA+CA
RT-
Agg/RA
RT-
Agg/-
RT-
P19
RT-
Liver
M
A
1444
955
736
476
341
258
105
HGF
(260bp)
B
β-actin
(330bp)

Chapter IV Characterization of The *Met*/Hepatocyte Growth Factor/Scatter Factor Receptor In Early Mouse Embryonic Development By Whole Mount *In Situ* Hybridization

Preface

Receptor tyrosine kinases are shown to play important roles in controlling cell growth and differentiation during embryonic development. Previous chapters have described the expression of the *met*/HGF/SFR in epithelial cells of many adult tissues, whereas HGF/SF is predominantly produced by mesenchymal cells, and suggest a role for the *met*/HGF/SFR in the interaction of mesenchymal and epithelial cells during embryogenesis. In addition, the detection of *met*/HGF/SFR expression in undifferentiated pluripotent teratocarcinoma P19 cells also suggests that the *met*/HGF/SFR may be expressed in embryos prior to organogenesis. The induction of a secondary neural tube by HGF/SFR in chick, and the expression of the *met*/HGF/SFR in P19 neuronal cell derivatives described in chapter III supports the involvement of the *met*/HGF/SFR in neurogenesis. To investigate the possible function of the *met*/HGF/SFR during embryogenesis, I chose to study the expression of the *met*/HGF/SFR in embryos from day 7.5-10.5 of gestation by whole mount *in situ* hybridization. This technique will allow visualization of the temporal and spatial expression of the *met*/HGF/SFR in the whole embryo. In chapter IV I demonstrate that expression of the *met*/HGF/SFR is not only localized to the epithelial precursors of tubulous organs and in a subpopulation cells of neuroepithelium, but also in potential migrating muscle precursor cells. These observations suggests that the *met*/HGF/SFR may play a role in cell proliferation, cell migration and tubulogenesis during embryogenesis.

Abstract

Hepatocyte growth factor/scatter factor and its receptor, *met*/HGF/SFR control proliferation, migration, and tubule formation of several epithelial and endothelial cell lines in culture. These are all processes that are important during embryonic development. Moreover, HGF/SF is primarily a mesenchymally derived factor, whereas expression of its receptor is restricted to epithelia in adult tissues. Since mesenchymal and epithelial cell interactions play an important role during embryogenesis, to investigate a possible role for the *met*/HGF/SFR in embryonic development, we have analyzed its expression in mouse embryos from day (D) 7.5 to 10.5 of gestation by whole mount *in situ* hybridization. *Met*/HGF/SFR expression is first detected in the ventrolateral portion of somites, C2-C4 in D9.25 mouse embryos, and is later localized to the ventrolateral portion of somites at regions where fore (C2-T1) and hind limb (L1-S2) buds form. At this stage *met*/HGF/SFR expression is restricted to a subpopulation of cells located at the ventrolateral portion of the dermamyotome whereas by D10.5 of gestation the *met*/HGF/SFR is expressed in cells that appear to migrate into dorsal and ventral regions of the limb buds. In addition expression of the *met*/HGF/SFR is also detected in the ventrolateral portions of the abdominal (T4-T12) and the caudal (L5-last) somites. During somite maturation, the expression of the *met*/HGF/SFR undergoes a remarkable redistribution between D9.25 and 10.5 of development, where in D10.5 mouse embryos the *met*/HGF/SFR is also expressed in cells located in the dorsal-medial region of the dermamyotome of somites from O3-L3. The localization of these cells corresponds to muscle precursor cells which are thought to migrate to form the axial and abdominal muscle primordium. In addition, to somites, the *met*/HGF/SFR is expressed in neuroepithelium, and epithelial precursor cells of primitive gut and nephrogenic cords of the D9 and 10 mouse embryos. These data support a role for the

met/HGF/SFR in cell migration, proliferation and tubulogenesis during embryogenesis.

Introduction

Hepatocyte growth factor (HGF), also known as scatter factor (SF) is a multifunctional cytokine with activities on a wide variety of normal and neoplastic cells. HGF/SF is a mitogen, dissociation and motility factor for many epithelial cells (Stoker and Gherardi, 1991; Matsumuto and Nakamura, 1993), stimulates invasion of carcinoma cells (Weidner et al., 1990) and tubulogenesis of tubular epithelial cells (Montesano et al., 1991). *In vivo* HGF/SF is a potent angiogenic factor (Chan et al., 1991) and is involved in organ regeneration (Matsumoto and Nakamura, 1993) and tumorigenesis (Rong et al., 1992). A high affinity receptor for HGF/SF has been identified as the product of the *met* proto-oncogene (Bottaro et al., 1991; Naldini et al., 1991), which encodes a receptor tyrosine kinase (RTK) originally isolated as an oncogene (*tpr-met*) (Cooper et al., 1984; Park et al., 1986). The mature form of the *met*/HGF/SFR is a heterodimeric protein of 190 kd, which consists of a 45 kd extracellular α-subunit linked by disulfide bonds to a 145 kd β-subunit (Gonzatti-Haces et al., 1988; Giordano et al., 1989; Rodrigues et al., 1991). The β-subunit spans the membrane and contains the catalytic kinase domain as well as a number of tyrosine phosphorylation sites (Giordano et al., 1989; Rodrigues et al., 1991). Upon stimulation with HGF/SF, the *met*/HGF/SFR β-subunit becomes phosphorylated on tyrosine residues (Bottaro et al., 1991; Naldini et al., 1991), which results in increased kinase activity of the receptor (Naldini et al., 1991). Activation of the *met*/HGF/SFR kinase is essential for the biological activities of HGF/SF in culture (Weidner, et al., 1993, Komada and Kitamura, 1993; Zhu et al., 1994).

Receptor tyrosine kinases (RTK) are recognized to play important roles in controlling cell growth and differentiation during embryonic development. Northern hybridization studies have demonstrated that the *met*/HGF/SFR and its ligand are expressed in the majority of adult mouse tissues (Chan et al., 1988; Iyer et al, 1990) and in undifferentiated pluripotent teratocarcinoma P19 cells

(Yang and Park, 1993). The biological activities of HGF/SF including mitogenesis, motogenesis and morphogenesis for epithelial cells are all processes involved in embryonic development. Moreover, HGF/SF is predominantely expressed in mesenchymal cells in culture (Stoker and Perryman, 1985; Rosen et al., 1989), and we have localized expression of its receptor to epithelial cells in adult mouse tissues (Yang and Park, 1994, submitted). Interactions between mesenchymal and epithelial cells, which are believed to be mediated mainly by diffusible factors and their receptors (Kratochwil, 1983), occur throughout embryonic development (Ekblom, 1989) and are crucial during gastrulation and organogenesis. The ectopic expression of the *met*/HGF/SFR and its ligand in NIH3T3 fibroblast cells is tumorigenic and results in partial conversion of mesenchymally derived cells to cells with epithelial characteristics (Tsarfaty et al., 1994). Moreover, ectopic expression of HGF/SF induces formation of neuronal tissues in the developing chick embryo (Stern, 1990). These observations, in addition to the multifunctional activities of HGF/SF *in vitro*, suggest that HGF/SF and its receptor may play a role in mesenchymal-epithelial cell interactions required during embryonic development.

To investigate the function of the *met*/HGF/SFR during early murine development, we have examined the temporal and spatial expression pattern of the *met*/HGF/SFR and its ligand in mouse embryos from D7.5 to 10.5 of gestation by whole mount *in situ* hybridization. We demonstrate that expression of the *met*/HGF/SFR is first detected in somites in D9.25 mouse embryos. We observe two distinct patterns of *met*/HGF/SFR expression in somites; the first at D9.25 of gestation (in embryos with 21-40 somites) in which expression of the *met*/HGF/SFR is localized to the ventrolateral portion of somites where the fore (C4-T1) and hind (L1-S2) limb buds are formed, and the second which starts at D10 of gestation where expression of the *met*/HGF/SFR is localized to the dorsal-medial portion of somites from O3-L3, as well as in the ventrolateral

region of abdominal (T4-T12) and caudal (L5-last) somites. This transition coincides with the identification of cells expressing *met*/HGF/SFR transcripts in the mesodermal core of the fore and hind limb buds. In addition to somites, *met*/HGF/SFR expression is also detected in neuroepithelium and the epithelial endoderm layer of the primitive gut and nephrogenic cords (the primitive urinary tract). These data identify *met*/HGF/SFR expression in epithelia that are undergoing proliferation, migration and tubulogenesis and support a role for the *met*/HGF/SFR in these processes.

Materials and methods

Mouse embryos and staging

Embryos were dissected from the F1 female mice of a B6 and C3 cross. Day 0.5 is scored as noon on the day plugs were identified. To allow a precise system of staging, embryos were viewed under a dissecting microscope and classified according to their somite numbers (Rugh, 1990).

Plasmids and riboprobe synthesis

As probes for hybridization studies, a 0.5 kbp EcoRI fragment (p*met* SC1) (Yang and Park, 1993) derived from the 5'-portion of the murine *met* cDNA (pcD) (Iyer, et al. 1990), or a 1.5 kbp EcoRI fragment (p*met* SC2) located at the 3' portion of p*met* SC1 were subcloned into the pBluescript KSII+ vector (Stratagene). A rat HGF/SF 1.4 kbp cDNA fragment including the 3'-portion of the α subunit, β subunit and 3' untranslated region was subcloned in pBluescript SK (-) (pRBC-1) (Tashiro et al., 1990). A 700bp Dra II/Hinc II or 260 bp Dra II/Hinc II cDNA fragment corresponding to the 3'-portion of the α subunit, β subunit was generated from the pRBC-1 clone and was subcloned into pBluescript SK (-) (Stratagene)

Non-radioactive antisense and sense riboprobes were synthesized by *in vitro* transcription using digoxigenin-UTP following the manufacturers instructions (Boehringer Mannheim; Munjaal, 1990). Probes in these experiments were as follows (Fig. 1): murine *met*/HGF/SFR antisense probes (Met1, corresponding to position 645-886 in the murine cDNA; Met2, position 434-886; Met3, position 2047-2469); and the HGF/SF antisense probes (rHGF, corresponding to position 1523-2150 in the rat cDNA; hHGF, position 418-673 in the human cDNA).

Whole-mount *in situ* hybridization

Whole mount *in situ* hybridization was performed according to a revised protocol kindly provided by Dr. Janet Rossant (Conlon et al., 1992). Embryos were dissected free in DEPC treated phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C with rocking. After washing three times, embryos were incubated for 1 hour with cold PBS containing 0.1% Tween 20 (PBT) at 4°C, then stored in 100% methanol at -20°C for less than a month.

All procedures were performed at room temperature with rocking unless noted. To block the endogenous peroxidase, embryos were treated with 5:1 methanol/30% hydrogen peroxide for 2h and rehydrated through a methanol/PBT series (75%, 50%, 25%). Following three washes with PBT, embryos were treated with 50µg/ml proteinase kinase K in PBT for 5 min. After washing twice with 2mg/ml glycine in PBT for 5 min and twice with PBT, embryos were refixed in fresh 0.2% glutaraldehyde/4% paraformaldehyde in PBS for 20 min. Following three washes with PBT, embryos were treated with fresh 0.1% sodium borohydride in PBT for 20 min without rocking and then washed three times with PBT. Embryos were prehybridized for at lease 1 hour at 65°C in hybridization buffer (50% formamide, 0.75M NaCl, 10mM PIPES pH 6.8, 1mM EDTA, 100µg/ml tRNA, 0.05% heparin, 0.1% BSA and 1% SDS). For hybridization, embryos were incubated at 65°C overnight in hybridization buffer with 0.4µg/ml digoxigenin labeled RNA probes.

Embryos were washed three times with wash 1 (300mM NaCl, 10mM PIPES pH 6.8, 1mM EDTA and 1% SDS), incubated in the same solution twice for 30 min each at 65°C with rocking, then washed twice and incubated twice for 30 min at 50°C with wash 1.5 (50mM NaCl, 10mM PIPES pH 6.8, 1mM EDTA and 0.1% SDS). Embryos were then treated with 100 µg/ml RNase A and 100U/ml RNase T1 in a buffer containing 0.5M NaCl. 10mM PIPES pH 7.2 and 0.1% Tween 20, then washed once with solution 2 (50% formamide, 300mM NaCl, 10mM

PIPES pH 6.8, 1mM EDTA, and 1% SDS), then incubated once with the solution 2 for 30 min at 50°C. Embryos were then washed with solution 3 (50% formamide, 150mM NaCl, 10mM PIPES pH 6.8, 1mM EDTA, and 0.1% Tween 20), and incubated for 30 min at 50°C in solution 3, washed with 500mM NaCl, 10mM PIPES pH 6.8, 1mM EDTA and 0.1% Tween 20, and placed at 70°C for 20 min.

To detect specific hybrids, embryos were incubated with TBST buffer (0.8g NaCl, 0.02g KCl, 0.3g Tris pH 7.6 and 0.1% Tween 20) plus 2mM levamisole and 10% heat-inactivated goat serum for at lease 1 hour at room temperature with rocking. Embryos were then incubated at 4°C overnight with 1/5000 anti-digoxigenin antibody coupled to alkaline phosphatase in cold TBST containing 2mM levamisole and 1% heat-inactivated goat serum. Antibodies were preabsorbed with heat-inactivated embryo acetone powder at 4°C for 1 hour and debris were removed by centrifugation. Embryos were washed three times with TBST plus 2mM levamisole and incubated six times, one hour each with the same solution at room temperature with rocking. After washing twice with freshly prepared NTMT (100mM NaCl, 100mM Tris pH 9.5, 50mM MgCl2, 0.1% Tween 20) plus 2mM levamisole, embryos were incubated overnight in the dark at room temperature with the same solution containing 337.5µg/ml nitrobluetetrazolium salt (NBT), 175µg/ml 5-bromo-4-chloro-3-indolyl-phosphate. The color reaction was stopped with three washes of CMFET (PBT plus 1mM EDTA and 0.1% Tween 20) and stored in CMFET or TBST at 4°C in the dark. Embryos were dehydrated through TBST/methanol series (30%, 50%, 75%, 100%). To decrease the background staining, some embryos were rehydrated and cleared by incubation with 50%, and then 80% glycerol/CMFET.

To determine the hybridization at the cellular level, stained embryos were re-fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, dehydrated with a TBST/methanol series (30%, 50%, 75%, 95% and 100% twice, 5 min each), and incubated with 100% methanol twice for 1 hour each, xylene twice

for 1 hour each, 1:1 xylene/paraffin twice for 30 min each at 60°C and paraffin three times for 1 hour each at 60°C. Embryos were then embedded in wax and sectioned at 7µm. Sections were dewaxed in 2 X 15 min in xylene and rehydrated in an ethanol series (100% X 2, 95%, 70%, 50% and water, 5 min each), followed by counterstaining with 0.002% eosin solution and mounted with Permount.

***In situ* hybridization**

Embryos were stored in liquid Nitrogen or directly mounted with OCT freezing medium. Sections were cut with a cryostat (Leitz) at 10µ and placed on poly-lysine coated slides. Sections were briefly air dried, then fixed in fresh 4% paraformaldehyde in PBS for 15 min at RT and washed twice with 1 x PBS, treated with proteinase K (10µg/ml) in 0.1M Tris-HCl pH 7.5, 50mM EDTA, 2mM $CaCl_2$ at 37°C for 10 min followed by incubation in 0.1M Triethanolamine containing 0.25% acetic anhydride for 10 min. For control slides, RNA was digested by treatment with 2µg/ml of RNase A and RNase T1 in 0.1M Tris, pH 7.5, 50mM EDTA and 4mM NaCl at 37°C for 1h prior to the proteinase K treatment. Slides were washed in 3 x PBS for 5min, 1 x PBS for 5 min and 0.2% glycine in PBS for 10 min, then prehybridized in a buffer containing 50% deionized formamide, 5 x SSC, 5 x Denhart's solution, 250µg/ml tRNA and 200µg/ml salmon sperm DNA at 45°C for at least 1h. Sections were dehydrated in alcohol prior to hybridization.

Antisense and sense riboprobes were diluted in prehybridization mix at 5ng/µl, and 20µl of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, then washed twice with 2 x SSC at 45°C for 15 min, and treated with 2µg/ml of RNase A at RT for 15 min. Slides were then washed once with 0.2 x SSC and twice with 0.1 x SSC at 45°C for 15 min each and blocked with 2% normal sheep serum and 0.3% triton X-100 in buffer 1 (100mM Tris-HCl. pH 7.5, 150mM NaCl) for 30 min. To detect

met/HGF/SFR specific hybrids, slides were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (1 to 5,000 dilution in buffer 1) for 30 min, then washed twice (15 min each) with buffer 1 and rinsed in buffer 3 (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM $MgCl_2$). The hybrids bound to anti-digoxigenin antibody can be visualized by a color reaction containing 337.5µg/ml nitrobluetetrazolium salt (NBT), 175µg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.24mg/ml Levamisole in buffer 3 and color was allowed to develop for 12 hours in the dark. The reaction was terminated by incubation with 100mM Tris-HCl, pH 8.0, 1mM EDTA for 5 min. Slides were dehydrated, incubated in xylene, mounted with permount and stored in the dark. Slides were viewed and photographed on a light microscope.

Results

Met/HGF/SFR transcripts are expressed in somites.

To investigate the involvement of the *met*/HGF/SFR and its ligand in early embryonic development, we have studied the expression of these genes in mouse embryos from D7.5 to 10.5 of gestation by whole mount *in situ* hybridization. At least twenty embryos for D9 and five embryos for other stages were hybridized with digoxigenin labeled *met*/HGF/SFR, or HGF/SF antisense or sense riboprobes (Fig. 1). Hybrids were detected using anti-digoxigenin antibody linked to alkaline phosphatase and visualized by a color reaction, with nitrobluetetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, which forms an insoluble blue precipitate. Embryos were dissected free from the uterus and staged according to the total somite number; D9 embryos are those with 13-20 somites; D9.25, 21-25 somites; D9.5, 26-30 somites; D9.75, 30-36 somites; D10, 36-40 somites and D10.5 over 40 somites (Table 1), which are classified as 4 occipital (O), 8 cervical (C), 12 thoracic (T), 5 lumbar (L), 5 sacral (S) somites and the rest are caudal somites (finally 29 by D16 of gestation) (Rough, 1976). A comparison of embryos hybridized with sense (Fig. 2A) and antisense *met*/HGF/SFR cRNA probes (Fig. 2B-H), revealed that specific *met*/HGF/SFR hybrids are located predominantly in somites in D9.25-10.5 embryos. Embryos hybridized with the sense *met*/HGF/SFR riboprobes do not show intense hybridization, but have a diffuse background staining throughout the embryo (Fig. 2A). Similarly, embryos hybridized with HGF antisense and sense riboprobes show diffuse staining throughout the embryo and specific hybridization could not be distinguished from background (data not shown). A Krox 20 riboprobe which hybridizes only to rhombomeres in embryos of D7 to D9 gestation was used as a positive control to confirm *met*/HGF/SFR specific hybridization (data not shown, Colon et al., 1992).

Whole mount *in situ* localization of *met*/HGF/SFR transcripts in developing somites.

Met/HGF/SFR transcripts are first detected in the 6th-7th somites (C2-C4) of D9.25 mouse embryos (21-25 somites) (data not shown), whereas no specific *met*/HGF/SFR expression was found in embryos that have less than 20 somites (data not shown). Mouse somites form by aggregation of dorsal mesodermal cells adjacent to the neural tube at D8 of gestation and develop in a rostrocaudal direction, where newly formed undifferentiated somites and fully differentiated somites can be found in the same embryo (Fig. 2C). In D9.25 to D9.5 embryos with 21-30 somites, *met*/HGF/SFR transcripts are localized to somites starting from the 6th somite (C2) and expression progresses towards the posterior (Fig. 2B-D, Table 1). In an embryo with 26 somites (approximately D9.5), *met*/HGF/SFR expression is restricted to 4 somites which show weak hybridization with a decrease in intensity in a rostrocaudal direction (C2-C5) (Fig. 2B). *Met*/HGF/SFR expression proceeds to 8 somites in an embryo with 28 somites (C2-T1) all of which now show strong staining with similar intensity (Fig. 2C). At this stage, *met*/HGF/SFR specific hybrids are located only at the ventral region of somites at the base of the fore limb bud, and not within the central (lateral) and dorsal region of somites (Fig 2C, D). The fore limb buds are formed at the region of somites C4 to T1 in an early D9 mouse embryo, whereas the hind limb buds develop at the level of somites L1 to S2 half day later (D9.5) (Carlson, 1981; Rugh, 1990). A similar pattern for *met*/HGF/SFR expression is observed in the hind limb bud region. By D10 *met*/HGF/SFR expression is localized in the ventral portion of somites (L1-L5) that line the base of the hind limb bud (Fig. 2F, G). At this stage no specific *met*/HGF/SFR hybrids are detected in the ventral region of the thoracic, T2- T12 somites (2F) or somites caudal to L5, suggesting that the *met*/HGF/SFR is only expressed in the ventral portion of somites at locations where the fore and hind limb buds are formed.

Interestingly, in embryos with approximately 36 somites (Fig. 2E), *met*/HGF/SFR transcripts are not only detected in the ventral region of somites C2-T1 and L1-S2, but are also detected at a low level in the dorsal region of the O3-C2 somites (Fig. 2E, H) and by D10.5, in embryos with approximately 45 somites (Fig. 3A, B), *met*/HGF/SFR hybrids are now detected in the dorsal portions of somites from O3 to L3 (Fig. 3B). At this stage *met*/HGF/SFR transcripts are no longer detected in the ventral region of somites C2-T1, and L1-L5 where the fore and hind limb buds are located (Fig. 3A, B, G). However *met*/HGF/SFR transcripts are now detected in the mesodermal core of both the fore and hind limb buds (Fig. 3D, F, G), with more intense and less diffuse staining in the fore limb buds (Fig. 3D).

In D10.5 mouse embryos *met*/HGF/SFR transcripts are localized in the dorsal region of somites from O3-L3 (Fig. 3A, B, G), as well as in the ventral region of the abdominal (T4-T12) and caudal (L5-last) somites (Fig. 3A, B, G). Thus we observe two distinct patterns of *met*/HGF/SFR expression in somites; the first at D9 of gestation (in embryos with 21-36 somites) where expression of the *met*/HGF/SFR is localized to the ventral portion of somites where the fore (C4-T1) and hind (L1-L5) limb buds are formed, and the second which starts at D10 of gestation where expression of the *met*/HGF/SFR is localized to the dorsal portion of somites from O3 to L3, and to the ventral region of the abdominal (T4-T12) and caudal (L5 to last) somites (Fig. 3A, B, G).

***In situ* localization of *met*/HGF/SFR transcripts to migratory cells in the limb buds**

To identify the cell types in somites that express *met*/HGF/SFR transcripts, embryos at various stages (D9.25 -10.5) following whole mount *in situ* hybridization, were fixed in paraformaldehyde and sectioned. In sequential sections of D9.5 embryos hybridized with *met*/HGF/SFR antisense riboprobes, intense staining is detected in cells corresponding to the dermamyotome within

somites (C2-T1) (Fig. 4A, B) and from transverse sections this is localized to a subpopulation of cells at the ventrolateral portions of somites where the body and limb junctions are located (C2-T1) (Fig. 4 C, D). By D9.75 *met*/HGF/SFR transcripts are no longer detected in the ventrolateral portions of somites (C4-T1), and this correlates with the presence of *met*/HGF/SFR specific hybrids in cells which are localized near the base of the fore limb bud (Fig. 4E) and eventually at the base of the fore limb bud (Fig. 4F). By D10.5, *met*/HGF/SFR expression is now detected in cells localized both to the dorsal and ventral portions of the fore (Fig. 4 G, H) and hind limb buds. In addition, corresponding with the switch of expression that we observe in whole embryos, upon sectioning D10.5 embryos, *met*/HGF/SFR transcripts are now detected in cells located in the dorsal-medial region of the dermamyotome of somites (Fig. 5A-C), but not in the myotome (Fig. 5A, B), and in the ventrolateral portions of somites in the trunk region (T4-T12) (Fig. 5D).

Localization of *met*/HGF/SFR transcripts in primitive tubular organs.

We have previously localized expression of the *met*/HGF/SFR in adult mice to luminal and surface epithelia in several tissues including kidney, lung, stomach, intestine and uterus (Yang and Park, 1994, submitted). Early in the period of organogenesis, at approximately D9, the intestinal and urinary tracts are essentially relatively simple tubes which differentiate rapidly (Carlson, 1981; Rugh, 1990). In both cases the surrounding mesenchymal cells promote both the intestinal and urinary tracts to differentiate from one layer of cells to complex tubular structures that give rise to duct forming organs such as kidney, liver and lung. To establish if, as suggested from *in vitro* tissue culture models, the *met*/HGF/SFR is involved in epithelial and mesenchymal cell interactions during organogenesis, we investigated *met*/HGF/SFR expression in the developing tubular structures that give rise to both the intestinal and urinary tracts.

Between D9-D10.5, the intestinal tract is formed by fusion of the fore- and hind-gut, followed by the differentiation of the pharynx, gastro-hepetic omentum and hind gut. The primordial digestive glands and respiratory system bud and branch from the foregut at late D9 following interactions between local gut endoderm and its surrounding mesoderm. Analysis of sections of D9.25 to 10.5 mouse embryos, previously subjected to whole mount *in situ* hybridization with *met*/HGF/SFR sense and antisense riboprobes, revealed a low level of *met*/HGF/SFR expression in the endodermal epithelial layer lining the intestinal tract (Fig. 6A, B, D, E). The *met*/HGF/SFR is expressed in the endoderm epithelial layer of foregut (Fig. 6A, B, D, E), and is also detected in the epithelia that bud from the foregut to form the trachea, lung and liver (Fig. 6D, E).

In D9 embryos, the nephrogenic cord is a newly formed simple tubule located in the cephalic portion of the intermediate mesoderm. Cells within the nephrogenic cord migrate caudally and gradually separate into the pronephros, mesonephros and metanephros (kidney analog) by late D10. Although the identification of these three structures in D9-10 embryos is rather difficult, a low level of expression of the *met*/HGF/SFR is detected in D9.5 embryos in mesodermal epithelial cells lining the newly formed nephrogenic cords (Fig. 6C, F). These results establish that the *met*/HGF/SFR is expressed in the early intestinal and urinary tubular precursor structures which later give rise to tubular organs, such as kidney, lung and stomach.

Localization of *met*/HGF/SFR transcripts to neuroepithelium.

In addition to expression in developing tubular epithelial structures, the *met*/HGF/SFR is also localized, by whole mount *in situ* hybridization, to neuroepithelium of the diencephalon and telencephalon in embryos at D9.5 of gestation (Fig. 6G, H). The neuroepithelium at this stage becomes greatly thickened by cell division of neuroblasts in the ependymal layer that line the

neural tube. Cells of the ependymal layer, which retain their epithelial character, are mitotic and eventually give rise to cells which migrate and differentiate into non-dividing functional neurons forming the marginal layer (Carlson, 1981; Uylings et al., 1990). Within this structure, *met*/HGF/SFR transcripts are localized to the marginal layer of the neuroepithelium adjacent to the optic vesicles (Fig. 6H), as well as in cells located in the basal layer of the retinal primordium in the optical vesicles. *In situ* hybridization was also done on frozen sections of D13.5 mouse embryos with digoxigenin-labeled *met*/HGF/SFR antisense riboprobes where we detected specific expression of the *met*/HGF/SFR in the root of spinal ganglia (Fig. 6I).

Discussion

Met/HGF/SFR expression and somite development.

The *met*/HGF/SFR tyrosine kinase, which is the receptor for HGF/SF, mediates the mitogenic, motogenic and tubulogenic responses to HGF/SF in epithelial cells in culture (Matsumoto et al., 1993, Stoker et al., 1987, Rosen et al., 1990, Montesano et al., 1991). All of these processes are essential during embryogenesis and to investigate the possible involvement of the *met*/HGF/SFR in early embryonic development we have studied its expression in mouse embryos from D7.5 to D10.5 of gestation by whole mount *in situ* hybridization. In this paper we show that *met*/HGF/SFR transcripts are not detected in D7 and D8 mouse embryos and are first identified in D9.25 embryos. At this stage expression of the *met*/HGF/SFR is localized to the ventrolateral region of the dermamyotome of somites (C2-T1) in the location where the fore limb buds are formed (Fig. 2C, D). Half day later (D9.75), *met*/HGF/SFR specific hybrids are now detected in the ventrolateral region of the dermamyotome of somites (L1-S2) where hind limb buds are formed (Fig. 2G). During embryogenesis, somites form following the condensation and conversion of dorsal mesodermal cells to an epithelial structure. Cells located in the medial region of the newly formed somite are the source of the myotome, which forms the axial, back and intercostal muscles whereas cells within the lateral portion of the somite migrate and give rise to ventral abdominal muscle and limb muscles (Emerson, 1993). Interestingly, the cells that expressed the *met*/HGF/SFR in the ventrolateral region of the dermamyotome, and later appear to migrate to the limb buds, seem to colocalize with muscle precursor cells (Fig. 2D, G; 3A, D, F) that migrate into the mesodermal core of the fore and hind limb buds as identified using chick/quail grafting and vital dye fluorescence labeling experiments (Christ et al., 1977, 1978, 1983, 1992; Kaehn et al., 1988; Hayashi and Ozawa, 1991, Ordahl and Le Douarin, 1992). These cells in the mouse are committed to a myogenic

lineage, although at this stage they do not express muscle lineage specific genes, including myf 4, myf 5, myogenin and Myo D (Sassoon et al., 1989; Ott et al., 1991; Cheng, et al., 1992; Sassoon, 1993; Yee et al., 1993).

The paired homeodomain containing gene, pax-3, is a possible molecular marker for the migratory dermamyotome precursor cells of limb bud muscle (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). Pax 3 expression colocalizes with the migrating cells. Moreover in a mouse mutant, splotch, the Pax 3 gene is mutated and the mice lack limb bud musculature (Epstein, et al., 1991; Franz et al., 1993a,b; Bober et al., 1994; Goulding et al., 1994). The expression pattern of the *met*/HGF/SFR, shows some features in common with pax-3 expression. The cells that expressed the *met*/HGF/SFR colocalize with pax-3 expressing cells in both the ventrolateral section of the somite and within cells that migrate into the limb bud. Based on this distribution, we believe that the *met*/HGF/SFR expressing cells correspond to myogenic precursor cells that migrate from the ventrolateral portion of the dermamyotome to form the limb bud musculature (Kaehn et al., 1988; Hayashi and Ozawa, 1991).

***Met*/HGF/SFR expression in somites is spatially and temporally regulated.**

During somite maturation, the expression of the *met*/HGF/SFR undergoes a remarkable redistribution between D9.25 and D10.5 of development, where in D10.5 mouse embryos the *met*/HGF/SFR is also expressed in cells located in the dorsal-medial region of the dermamyotome of all somites from O3-L3 (Fig. 3A, B). The localization of these cells corresponds to muscle precursor cells which are thought to migrate to form the axial muscle primordium (Ordahl and LeDouarin, 1992). Within developing somites the dorsal-medial area adjacent to the neural tube is thought to give rise to the axial muscles, whereas the lateral region of the somite is though to give rise to the limb and ventral abdominal musculature (Christ et al., 1983; Ordahl and Le Douarin, 1992; Emerson, 1993). Cells from the

dorsal portion of the somite also form the dermatome, an epithelial structure that arises as the somite develops and then disperses to give rise to cells of the dermis (Christ et al., 1992). We have localized *met*/HGF/SFR expression to the dermamyotome or dermatome. Whether these cells contribute to form the structure of axial muscles or dermis remains to be investigated. However these results, together with the observation that the *met*/HGF/SFR is expressed in muscle of the intercostal region and the mandible of embryos between D11 and D15 of gestation (Sonnenberg et al., 1993), suggests that the dorsal-medial dermamyotome cells expressing the *met*/HGF/SFR may be involved in the formation of intercostal and back muscle.

The fact that the *met*/HGF/SFR is not expressed in all cells of the somite, but specifically in cells that correspond to the migratory muscle precursor cells suggests that the *met*/HGF/SFR is not a general marker for somite epithelium. Moreover the detection of *met*/HGF/SFR expression in limb bud and mandible (Sonnenberg, et al., 1993), in cells which overlap with those expressing myogenin in D11 to D13 embryos (Sassoon et al., 1989), and the fact that low levels of HGF/SF specific transcripts were detected in the limb bud in D11 to D15 mouse embryos (Sonnenberg et al 1993), supports the hypothesis that the migrating muscle cells express the *met*/HGF/SFR. Interestingly the *met*/HGF/SFR is also expressed in muscle derivatives which differentiate in cultures of pluripotent P19 teratocarcinoma cells following treatment in culture with DMSO (Yang and Park, 1993). However, the *met*/HGF/SFR is not expressed in terminally differentiated skeletal muscles in the adult animal (Yang and Park, 1994), suggesting that expression of the *met*/HGF/SFR is required only in differentiating, proliferating and migrating muscle precursor cells.

***In situ* localization of the *met*/HGF/SFR in the primitive gut and nephrogenic cords.**

HGF/SF induces canine kidney epithelial cells (MDCK) cultured in a collagen matrix to form branching tubules (Montasseno et al., 1991, Santos et al., 1994), suggesting that the *met*/HGF/SFR and its ligand may be involved in inductive interactions during embryogenesis required for the formation of tubular organs. *Met*/HGF/SFR transcripts are detected in the endodermal layer of primitive gut and epithelia of the nephrogenic cords (primitive urinary tract) in D9 and D10 embryos. At these stages the primitive gut and nephrogenic cords are only simple tubules with a single layer of endodermal or epithelial cells that eventually give rise to tubular organs. During development, interactions with adjacent mesodermal cells stimulates both cell proliferation and migration of the gut endothelium and as a consequence the primitive gut becomes divided anteroposteriorly and the primordial digestive glands and respiratory system develop following subsequent mesenchymal inductive events (Carlson 1981). Similarly the nephrogenic cords (the primitive urinary tract) also undergoes a developmental pattern in which the endothelium thickens, proliferates, migrates, and follows a transition from mesenchyme to epithelia, and eventually becomes the pronephric tubule, mesonephros and metanephros (Carlson 1981). Although the *met*/HGF/SFR is expressed throughout the respiratory epithelia and that of the gut and intestine in developing (Sonerberg, et al., 1993) and adult organs (Yang and Park, 1994) it shows a restricted expression to the proximal and distal tubular epithelial cells of the developing (Soneberg et al., 1993) and adult kidney (Yang and Park, 1994) suggesting again that the *met*/HGF/SFR is not a general marker for epithelia in these tissues. The observation that the *met*/HGF/SFR is expressed in the single layer of tubular endothelium of the primitive gut and urinary tract suggests a possible role for the *met*/HGF/SFR in the proliferation, migration or morphogenesis of these epithelial stem cells, whereas in the adult animal the *met*/HGF/SFR may be involved in similar in cellular events, involved in tissue repair, architecture and homeostasis.

Localization of *met*/HGF/SFR transcripts in neuronal cells.

The *met*/HGF/SFR is also expressed in neuroepithelium localized to the marginal layer in the diencephalon and telencephalon in mouse embryos at D9 and 10 of gestation. At later stages of development *met*/HGF/SFR transcripts are detected in the ventral horns of the spinal cord in the telencephalon of D11 mouse embryos (Sonneberg et al., 1993). This corresponds with the formation of motoneurons and suggests that the cells that expressed the *met*/HGF/SFR in the marginal layer of the neuroepithelium of the diencephalon and telencephalon in the younger embryos may be progenitors for motoneurons. In support of a possible role for the *met*/HGF/SFR in neuronal cell migration or differentiation, the *met*/HGF/SFR is expressed as pluripotent P19 teratocarcinoma cells are induced to differentiate into neurons (Yang and Park, 1993) and cells enriched in HGF/SF when grafted into chicken embryos induce the formation of a secondary neural tube (Stern et al., 1990).

In general *met*/HGF/SFR expression is localized to epithelial structures in early mouse embryos however the restricted expression profile during somite differentiation and in neuroepithelium does not support a general role for the *met*/HGF/SFR in epithelia but instead suggests a specific role for this receptor in these structures. The *met*/HGF/SFR is expressed in epithelia that have the capacity to migrate, proliferate and form tubular structures. These correlate with the activities of HGF/SF in culture and supports a role for the *met*/HGF/SFR in these processes *in vivo*. A detailed molecular investigation using techniques that alter *met*/HGF/SFR expression during embryogenesis would be required to establish the exact function of the *met*/HGF/SFR in these processes during embryonic development.

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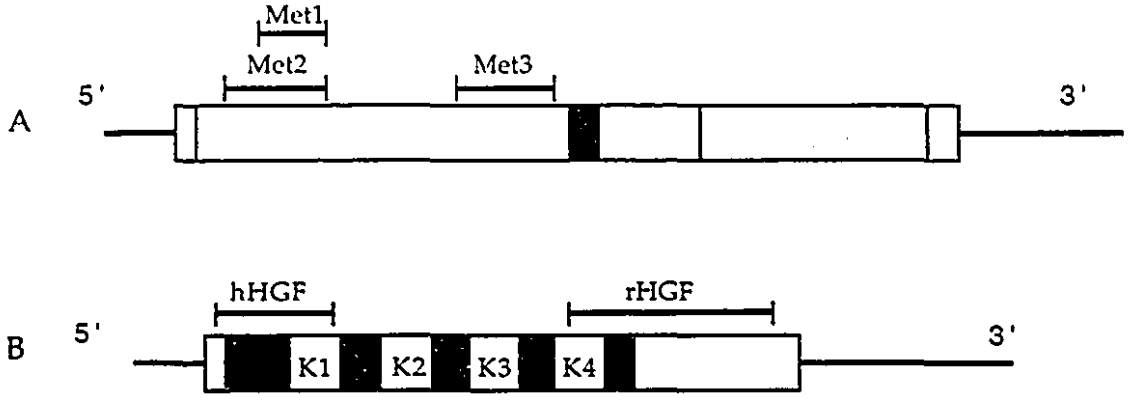

Figure 1. Schematic of the *met* /HGF/SFR and HGF/SF cDNAs.

The translated sequences are shown by boxes. The cRNA probes for *in situ* hybridization (Met 1, Met 2, Met 3 or hHGF, rHGF) are indicated. A. *met*/HGF/SFR cDNA, transmembrane domain and kinase domain are indicated as solid black box and striped box respectively. B. HGF/SF cDNA. K1-K4 show the four kringle structures of the HGF/SF.

Figure 2. Expression of *met*/HGF/SFR in somites visualized by whole mount *in situ* hybridization.

Mouse embryos hybridized with sense (A, 28 somites) or antisense *met*/HGF/SFR (B-H). At D9.25 (B) arrowhead points at specific *met*/HGF/SFR hybrids in the ventral regions of the C2 somites. At D9.5 (C & D) arrowheads mark hybrids in the ventral portions of somites at the base of the fore limb bud. At D9.75 (E) arrowheads shows hybrids in the ventral portions of somites. At D10 (F-H) arrowheads point to the hybrids in the ventral regions of somites at the base of hind limb bud (F, G), and no specific hybridization is detected in the somites between the two limb buds. (H) Arrowheads show hybrids in the ventral and dorsal regions of somites.

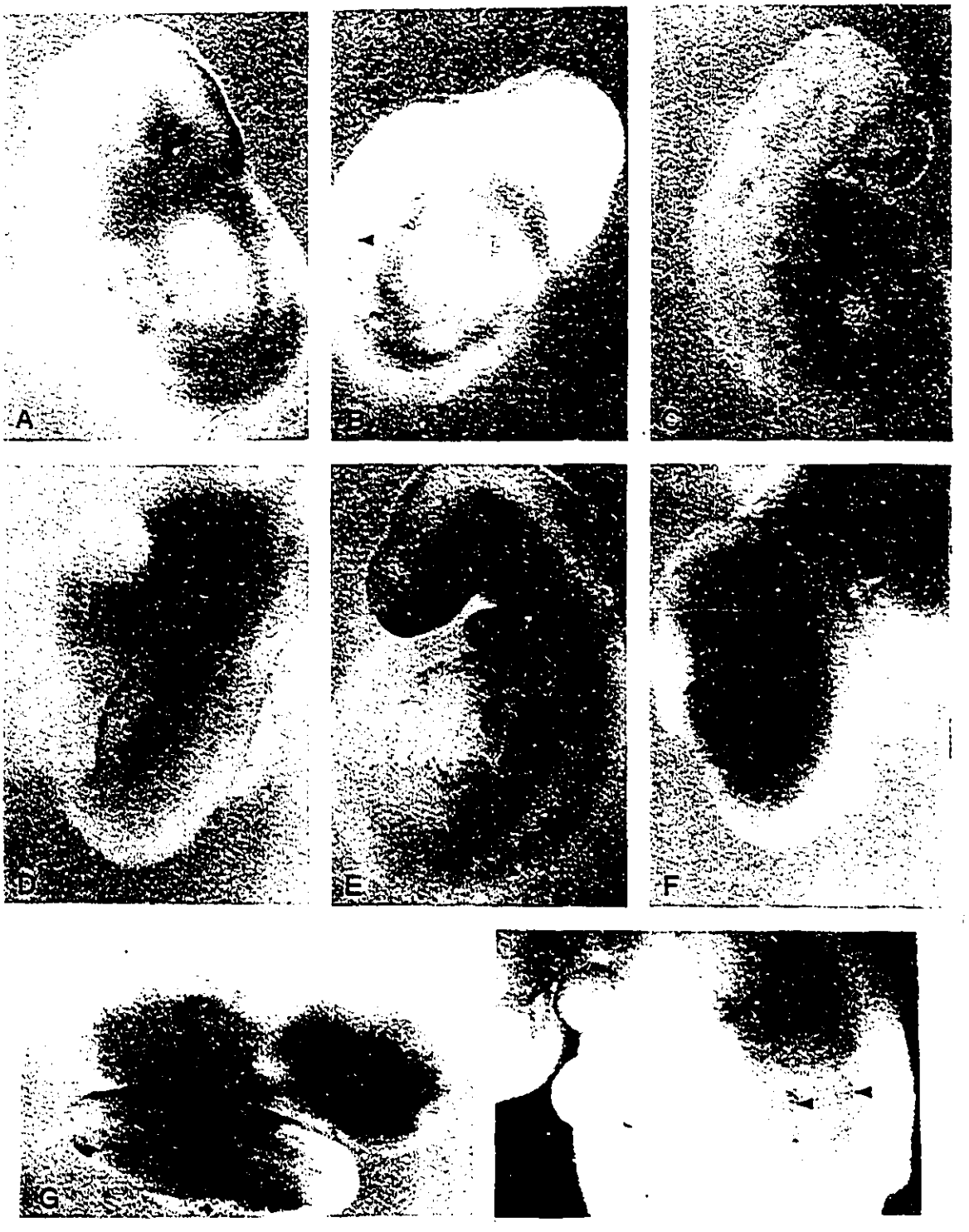
A
B
C
D
E
F
G

Figure. 3. Redistribution of the *met*/HGF/SFR expression in somites of D10.5 mouse embryos.

Hybridization with antisense (A, B, D, F, G) and sense (C, E) *met*/HGF/SFR riboprobes. Specific *met*/HGF/SFR hybrids represented as blue precipitates are detected in the dorsal regions of somites from O3 (A) to L3 (B, G), and in the ventral regions of somites from T4-T12 (A, B) and somites from L5 to tail (B, G). An intense signal is detected in the mesodermal core of the fore limb bud (A, B, D), whereas a more diffuse signal is observed in the hind limb bud (F, G).

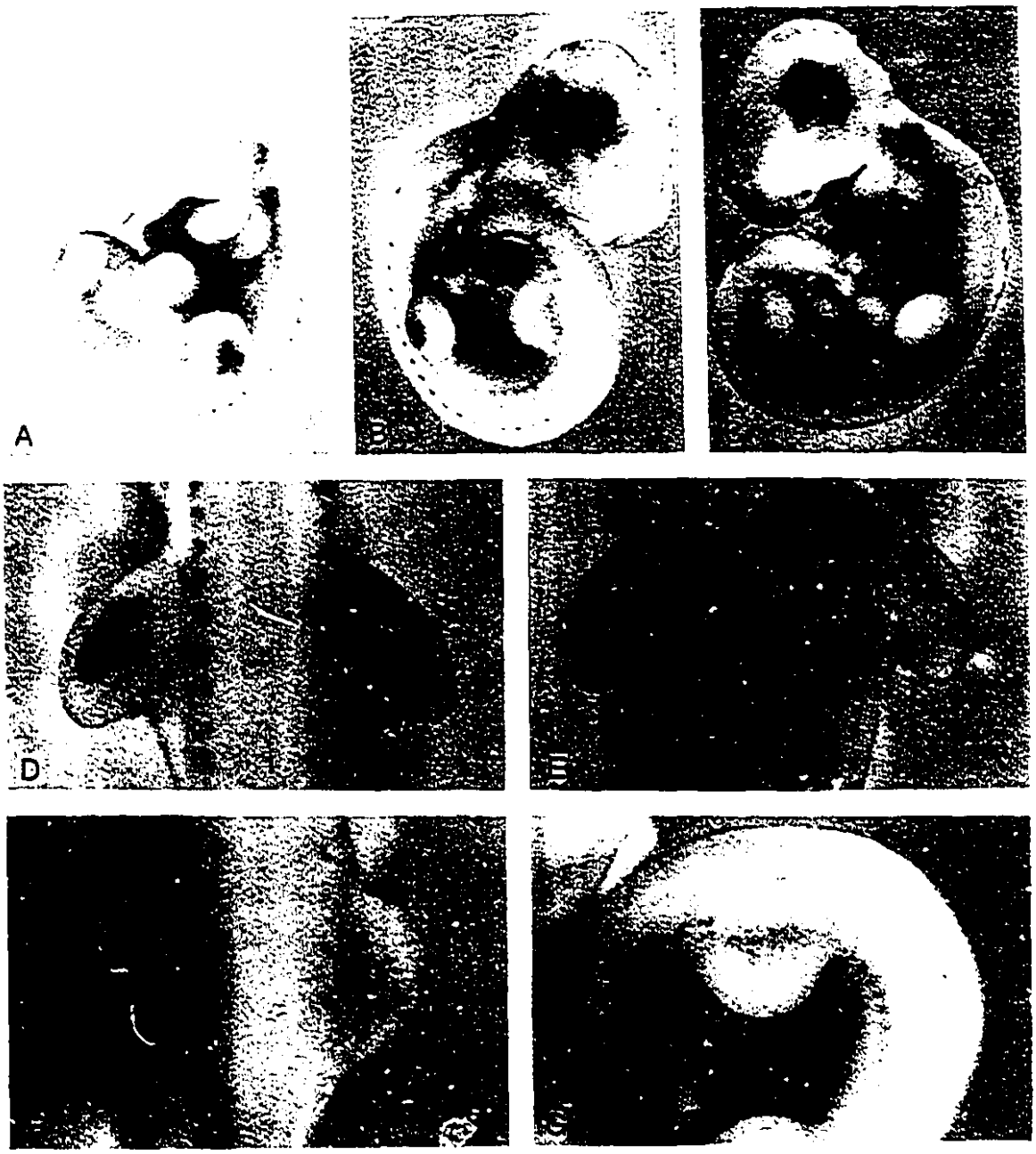
A
D

Figure. 4. Cells that expressed the *met*/HGF/SFR migrate into the limb bud.

At D9.25-9.75, the expression of the *met*/HGF/SFR (indicated by arrowheads) is detected in a subpopulation of cells located at the ventrolateral regions of dermamyotome (A or B high magnification) in the body-limb junction (C or D high magnification), and these cells appear to migrate towards the limb bud (E) and finally reach the base of the limb bud (F). At D10.5 the *met*/HGF/SFR specific hybrids are now detected in the dorsal and ventral mesoderm of the limb bud (G or H high magnification). * marks the limb buds.

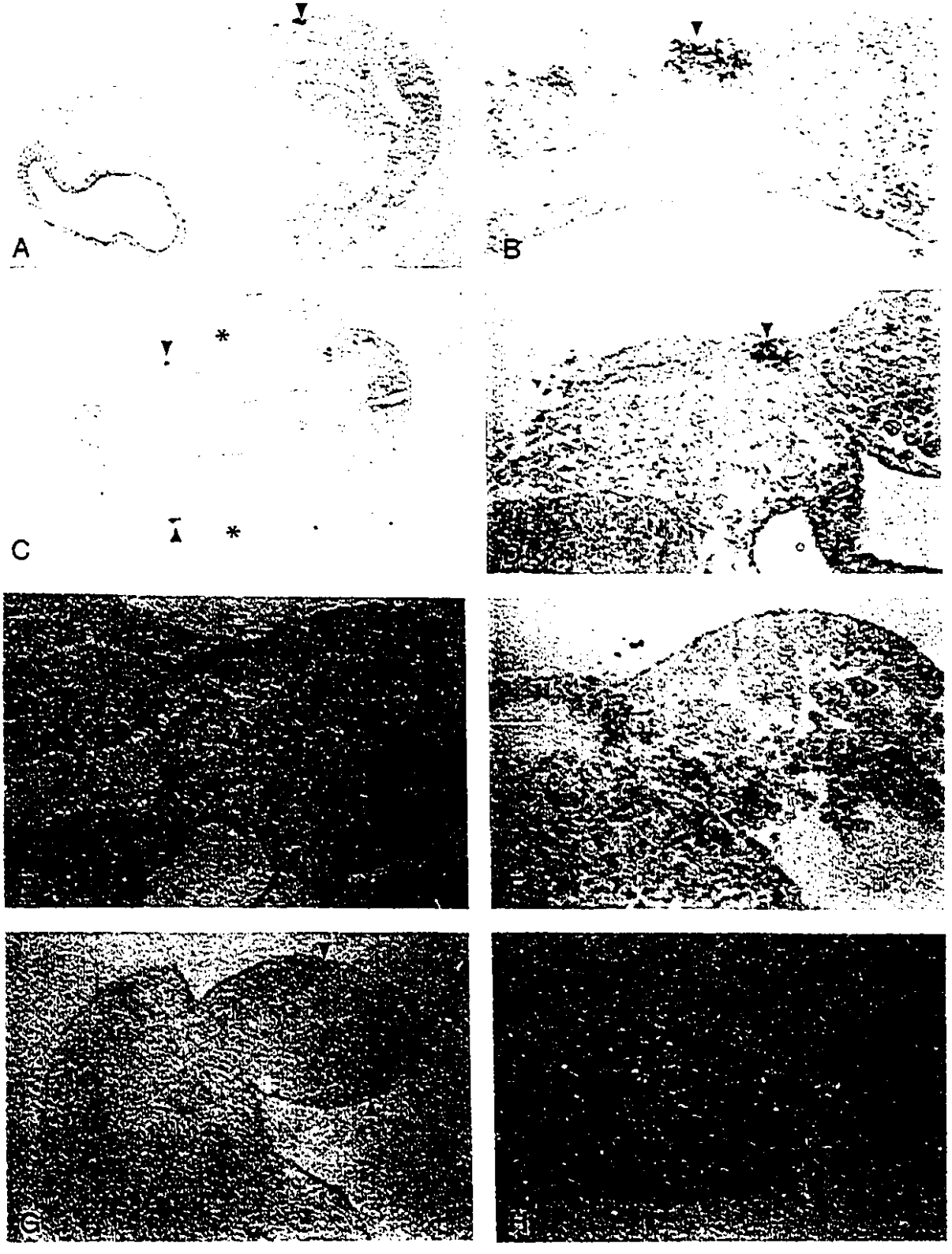
A
B
C
*
*

Figure 5. Expression of the *met*/HGF/SFR in D10.5 mouse embryos.

Specific *met*/HGF/SFR hybrids (indicated by arrowheads) are detected in the dermamyotome (A or B high magnification), but not in the myotome (arrow), and are located in the dorsal-medial regions of the somite (C). Hybridization signals are also detected in ventrolateral reions of the abdominal somites between the hind and fore limb bud (D). * shows the limb bud.

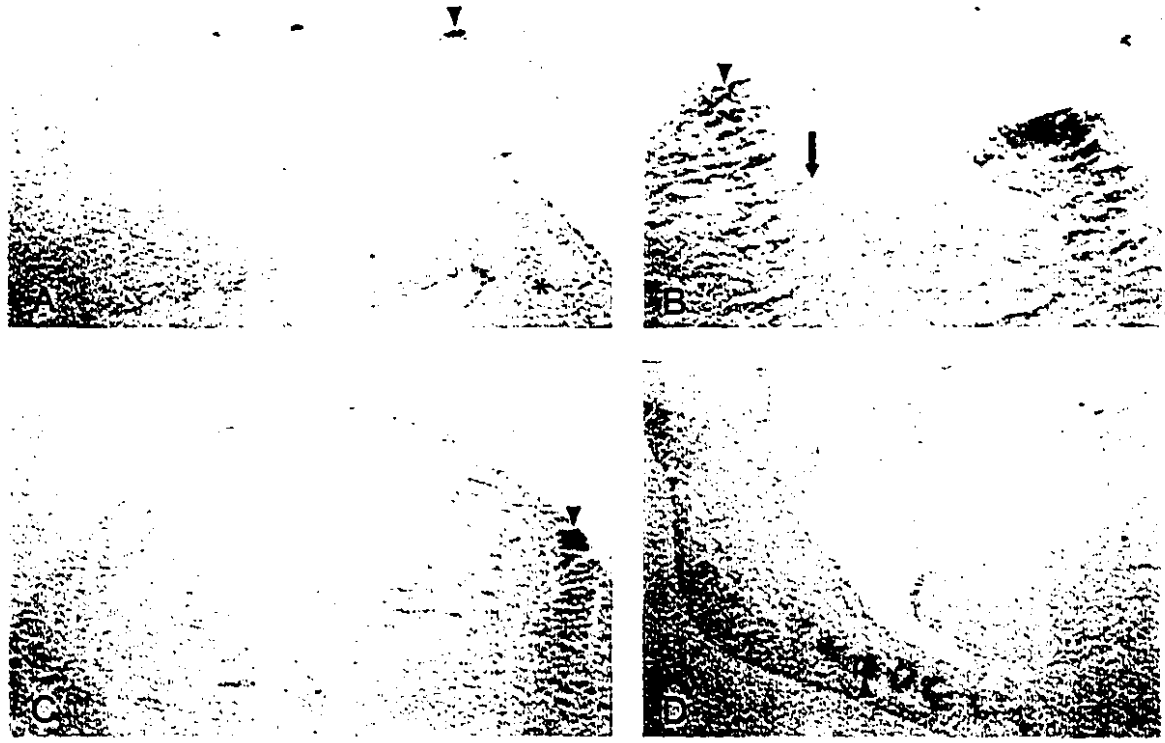
A
B
C
D

Figure. 6. Expression of the *met*/HGF/SFR in epithelial and neuronal cells.

Specific *met*/HGF/SFR hybrids (arrowheads) are detected in epithelial cells of the foregut (A or B high magnification), including those epithelial cells that surround the lung bud (D), as well as in a subpopulation of cells within the liver bud (E). The nephrogenic cord including the pronephric and mesonephric tubules shows low level of *met*/HGF/SFR expression (arrowhead, C & F). Hybridization is also detected in cells located in the maginal layer of the neuroepithelium in the diencephalon (d in G & H) and in the basal layer of the retinal primordium of the optical vesicles (o in G & H), as well as in the root of spinal ganglia of the D13.5 mouse embryo detected by *in situ* hybridization (I).

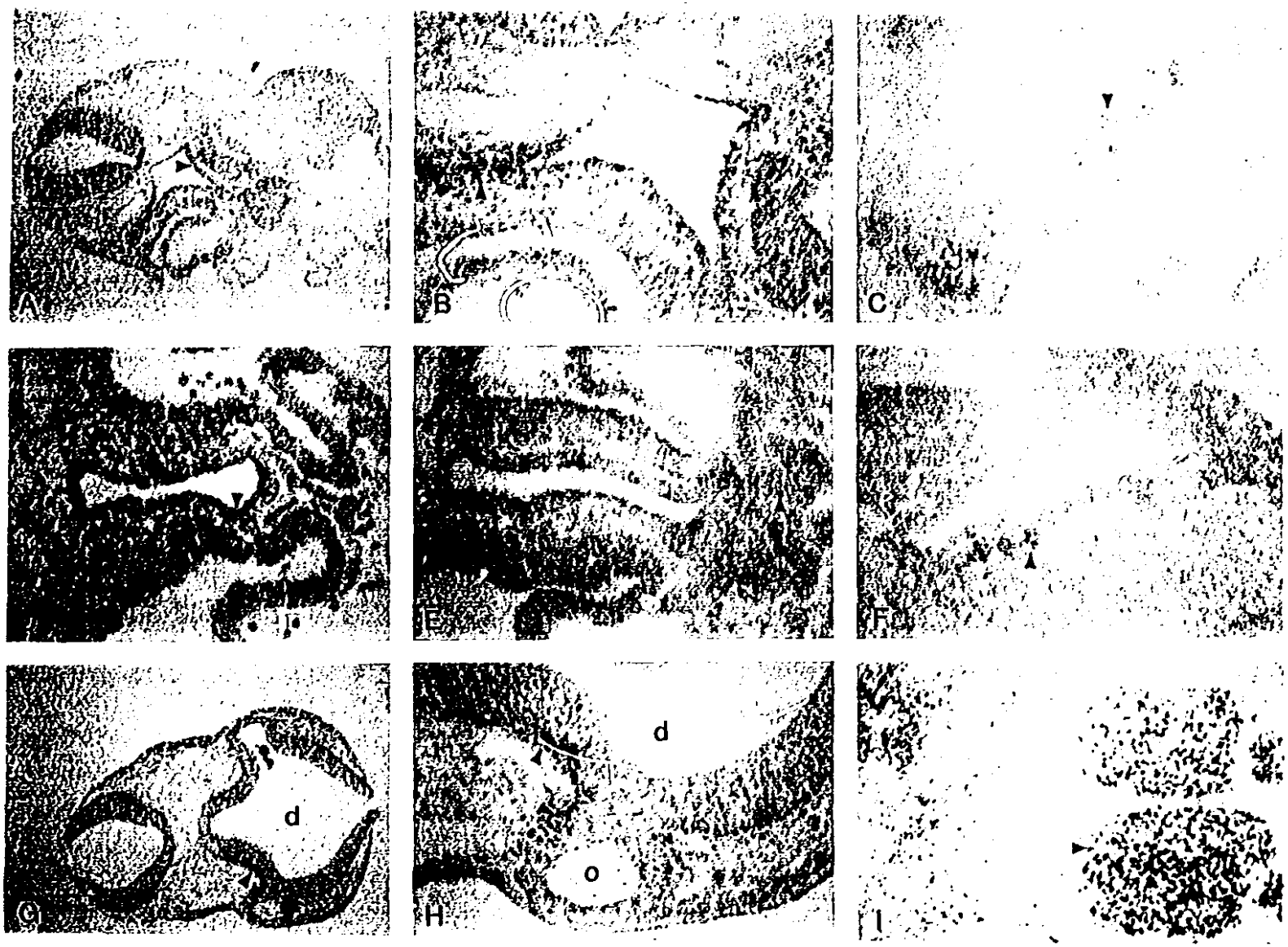
A
B
C
D
E
F
G
d
H
d
o
I

Table 1. Expression of the *met*/HGF/SFR in somites

Embryonic stage	Total somites	Hybridization		
		Somite #	Position in Somites	Location
D9	<20		-	
D9.25	21-25	C2-C4	Ventral-lateral	
D9.50	26-30	C2-T1	Ventral-lateral	Fore limb bud
D9.75	30-36	C2-T1	Ventral-lateral	Fore limb bud
		O3-C1	Dorsal-medial	
D10	36-40	C2-C3	Ventral-lateral	
		C4-T1	-	Mesodermal core of fore limb bud
		O3-C2	Dorsal-medial	
		L1-L5	Ventral-lateral	Hind limb bud
D10.5	>40	T4-T12	Ventral-lateral	
		L5-Cl	Ventral-lateral	
		O3-L3	Dorsal-medial	
		C4-T1	-	Mesodermal core of fore limb bud
		L1-L5	-	Mesodermal core of hind limb bud

* C, Cervical; T, Thoracic; O, Occipital; L, Lumbar; S, Sacral; Cl, Caudal.

Chapter V General Discussion

Binding of RTKs to polypeptide growth factors activates signal transduction pathways that control a range of cellular activities, including proliferation, migration, and differentiation. The expression of RTKs and their ligands is regulated in a tissue and cell-type specific manner, and are involved in many processes required for normal embryonic development and tissues homeostasis. Alterations in gene expression of RTKs and their ligands can result in aberrant cell growth manifested as tumorigenesis, and abnormal development. Examination of the cell-type specific expression of RTKs and their growth factors has given insights into their normal physiological functions. The *met* gene, which was recently identified as the RTK for hepatocyte growth factor (HGF) and scatter factor (SF), mediates mitogenesis, motogenesis, and tubulogenesis of epithelial and endothelial cells in culture (Bottaro et al., 1991; Naldini et al., 1991, Stoker and Gherardi, 1991; Weidner et al., 1990; Montesano et al., 1991, Matsumoto and Nakamura, 1993). The aim of this thesis project was to examine the possible physiological function of the *met*/HGF/SFR by identifying the specific cell types in which it is expressed in adult mouse tissues and during embryogenesis using *in situ* hybridization techniques.

The *met* gene was originally isolated as an oncogene, *tpr-met* in a MNNG-treated HOS cells (Cooper et al., 1984; Park et al., 1986). The *met*/HGF/SFR gene is amplified and overexpressed in several gastric tumor cell lines including GTL-16, MKN 45 and Okajima, in primary gastric tumors and in spontaneously transformed NIH 3T3 cells, and is overexpressed in many human colorectal carcinomas (Giordano et al., 1989, Liu et al., 1992, Tsao et al., 1993). In addition HGF/SF promotes the progression of carcinoma cells to a more invasive phenotype (Weidner et al., 1990, Jiang et al., 1993). Therefore HGF/SF and its receptor could be determinant in pathological processes such as cancer and

metastasis. However, little is known about the normal physiological function of the *met*/HGF/SFR *in vivo*, and therefore to provide some insights for its function it was important to understand the normal expression pattern of the *met*/HGF/SFR.

The study in chapter II demonstrates that the *met*/HGF/SFR is expressed in hepatocytes of resting liver, and is predominantly localized to tubular epithelia that line the proximal and distal convoluted tubules, Bowman's capsule of the kidney, and surface epithelia of stomach, oesophagus, lung and uterus and skin, as well as in granulosa cells of maturing and mature oocytes. By reverse transcriptase polymerase chain reaction amplification I have shown that the HGF/SF gene is expressed at low levels in many of these tissues. These observations support data from cell culture studies, where the *met*/HGF/SFR mediates mitogenesis, motogenesis and tubulogenesis predominantly in epithelial cells in response to treatment with HGF/SF (Stoker & Gherardi, 1991; Matsumoto & Nakamura, 1993; Montesano et al., 1991). HGF/SF is predominantly expressed by mesenchymal and stromal cells in culture (Stoker & Perryman, 1985; Rosen et al., 1989), and therefore may activate the receptor in a paracrine mode *in vivo*. These observations suggest that the activation of the *met*/HGF/SFR by its ligand may be involved in epithelial-mesenchymal interactions that occur normally during tissue organization, embryogenesis and organogenesis.

One feature for maintenance of tissue homeostasis is tissue regeneration after injury and wound healing. HGF/SF was purified from the plasma of patients with fulminant hepatic failure, and shown to be the most potent mitogen for mature hepatocytes in primary culture (Gohda et al., 1988). Therefore HGF/SF is thought to play an important role for liver regeneration following hepatic injury. HGF/SF was also detected in the plasma of rats with various organ injuries. HGF mRNA was markedly increased prior to the onset of renal

tubular cell replication after renal injury (Igawa et al., 1992). In addition, when HGF/SF was intravenously injected into mice subjected to unilateral nephrectomy, it stimulated DNA synthesis of renal tubular epithelial cells and suppressed the onset of severe renal failure, suggesting that HGF/SF may be a potent renotropic factor for renal regeneration (Matsumoto and Nakamura, 1993). Moreover, surface epithelia are labile structures and require frequent renewal though mitosis. HGF/SF is a mitogen for many epithelial cells in culture (Matsumoto and Nakamura, 1993), therefore, the detection of *met*/HGF/SFR expression in luminal epithelial cells of adult tissues suggests that one function of activation of the *met*/HGF/SFR is to stimulate the proliferation and renewal of surface epithelial cells. Based on the findings that HGF/SF and its receptor are present in a wide variety of tissues, and the *met*/HGF/SFR mRNA is localized predominantly in epithelial cells of tissues including kidney, lung, stomach, intestine, uterus and ovary, it supports a role for the *met*/HGF/SFR and its ligand, HGF/SF in epithelial cell proliferation and therefore play important roles in both regeneration and homeostasis of these tissues.

HGF/SF is not only a mitogen for a broad spectrum of epithelial and endothelial cells in culture (Matsumuoto and Nakamura, 1993), but also stimulates the dissociation and motility of epithelial and endothelial cells (Stoker and Gherardi, 1991), and acts as a morphogenic factor that stimulates the formation of branching tubules from tubular epithelial cells cultured in a collagen matrix (Montesano et al., 1991). Cell scattering and tubular morphogenesis observed *in vitro* may belie important activities that take place *in vivo*. Tissue regeneration takes place as cells are replaced through mitosis and requires movement in order to maintain the normal tissue architecture (Cunha, G.R. 1985). The cellular responses of epithelia to HGF/SF treatment and the expression of the *met*/HGF/SFR in epithelial cells *in vivo* supports a role for the *met*/HGF/SFR and its ligand in epithelial cell growth and tissue organization.

Since much of the control of epithelial cell proliferation and migration during embryogenesis is thought to be mediated by diffusible factors produced by mesenchymal cells, the *met*/HGF/SFR may be involved in cell differentiation and development during embryogenesis. The normal *met*/HGF/SFR gene appears to be ubiqutiously expressed in adult mouse tissues and in several mouse embryonic and extra-embryonic tissues by day 12-18 of gestation (Chan et al., 1988; Iyer et al., 1990). To understand a possible function for the *met*/HGF/SFR and its ligand, one approach is to identify the specific cell types which express the *met*/HGF/SFR and its ligand.

To study the possible involvement of the *met*/HGF/SFR in cell differentiation, I first chose to use the P19 mouse embryonic carcinoma (EC) cells, which are derived from a teratocarcinoma and are thought to correspond to the inner cell mass of the preimplantation embryo (McBurney et al., 1982). P19 cells are pluripotent and can be induced to differentiate into neuronal and mesodermal derivatives. Following treatment with retinoic acid (RA), P19 EC cells differentiate into neurons, astrocytes and fibroblast-like cells, and treatment with dimethyl sulfoxide (DMSO) can induce P19 cells to differentiate into skeletal muscle, cardiac muscle, as well as extraembryonic endothelial cells (Rudnicki and McBurney, 1987). The study in chapter III demonstrated that the *met*/HGF/SFR and its ligand are expressed at a low level in undifferentiated P19 cells and in cell aggregates treated with RA or DMSO, suggesting that the *met*/HGF/SFR and its ligand may be expressed in embryos prior to organogenesis. When cells were differentiated following treatment with RA or DMSO, the expression levels of the *met*/HGF/SFR and its ligand are increased. Although all cell types that expressed the *met*/HGF/SFR were not identified, the *met*/HGF/SFR mRNA and protein are expressed in neuronal cells. Moreover, the *met*/HGF/SFR is expressed in a skeletal myoblast cell line, C2C12, suggesting that skeletal muscle cells in the DMSO treated P19 cells may express the *met*/HGF/SFR. However, other

differentiated cell types among the P19 aggregates may also express the *met*/HGF/SFR. Ectopic expression of tyrosine kinases can induce P19 cells to differentiate into neurons. For example stimulation of P19 cells that express the EGFR cDNA with EGF (den Hertog et al., 1991), or expression of v-Src induce differentiation of P19 cells into neurons without RA treatment (Lynch et al., 1986). The *met*/HGF/SFR is the only RTK so far shown to be expressed in undifferentiated P19 cells. In addition, the fact that a second neural tube and primitive streak were induced when cells enriched in HGF/SF were grafted into chick embryos (Stern et al., 1990), plus the expression of the *met*/HGF/SFR in the neuroepithelium of embryos stated in Chapter IV, support a possible role for the *met*/HGF/SFR in the control of neuronal cell differentiation.

To analyze the functions of the *met*/HGF/SFR in mammalian development, I have characterized the expression of the murine *met*/HGF/SFR by whole mount *in situ* hybridization in embryos of day (D) 7.5 to 10.5 of gestation, reported in chapter IV. Expression of the *met*/HGF/SFR is first detected in somites of embryos at D9.25 gestation. At this stage expression of the *met*/HGF/SFR is localized to the ventrolateral region of the dermamyotome of somites in the location where the fore limb buds are formed (Fig. 2C & Fig. 3F in chapter IV). Half day later (D9.75), *met*/HGF/SFR specific hybrids are now detected in the ventrolateral region of the dermamyotomes of somites at regions where hind limb buds are formed (Fig. 2G in chapter IV). During embryogenesis, all of the skeletal muscles arise from somites which are formed following condensation of dorsal mesodermal cells and conversion to an epithelial structure. Cells located in the medial region of the newly formed somite are the source of the myotome, which forms the axial, back and intercostal muscles. Lateral somite cells migrate from the somite to give rise to the ventral abdominal muscle and limb muscles (Emerson, 1993). Interestingly, the cells that expressed the *met*/HGF/SFR in the ventrolateral region of the dermamyotome, and later

migrate to the limb buds appear to colocalize with muscle precursor cells (Fig. 2 & 4 in chapter IV) that migrate into the mesoderm core of the fore and hind limb buds as identified using chick/quail grafting experiments (Christ et al., 1977, 1978, 1983, 1992; Kaehn et al., 1988; Hayashi and Ozawa, 1991, Ordahl and Le Douarine, 1992). Moreover, the *met*/HGF/SFR is also expressed in cells located in the dorsal-medial region of the dermamyotome of all somites in D10.5 embryos (Fig. 3 & 5 in chapter IV). The localization of these cells corresponds to muscle precursor cells which are thought to migrate to form the axial muscle primordium (Ordahl and Le Douarin, 1992). The fact that the *met*/HGF/SFR is not expressed in all cells of somite, but specifically in cells corresponding to the migrating muscle precursor cells suggests that the *met*/HGF/SFR may play a specific role in initiation of limb and axial muscle differentiation or possibly in motility of cells involved in the formation of limb and axial muscles. Moreover the detection of *met*/HGF/SFR expression in limb bud and mandible (Sonerberg, et al., 1993), in cells which overlap with those expressing myogenin in D11 to D13 embryos (Sassoon et al., 1991), supports the hypothesis that the differentiating muscle cells express the *met*/HGF/SFR. However, in the adult animal, the *met*/HGF/SFR is not expressed in skeletal muscles which have terminally differentiated (Chapter II), suggesting that the expression of the *met*/HGF/SFR is present only in differentiating and migrating muscle precursor cells. The *met*/HGF/SFR is also expressed in muscle derivatives in DMSO-treated P19 cells and in a skeletal muscle cell line, C2C12. All together, these observations suggest that the *met*/HGF/SFR may play a role in muscle cell differentiation or migration.

The formation of skeletal muscle during embryogenesis involves commitment of mesodermal progenitors to the myogenic lineage and subsequent differentiation of skeletal myoblasts into a terminally differentiated myotube (Emerson, 1993). The activation of muscle specific genes, such as the *Myo* D gene

family, and initiation of differentiation in the myotome and in the limb bud is so rapid (within 12 hours), that it is thought that the initiation of muscle differentiation is not proceeded by proliferative expansion of a population of myoblast progenitors. Instead, the muscle differentiation is though to initiate from the migration of the muscle precursor cells located at the lateral and medial regions of the dermamyotome of somites (Emerson, 1993). However, to choreograph the formation of skeletal muscle, it requires an understanding of the cellular and molecular events occur during the formation of skeletal muscle. Interestingly the expression of the *met*/HGF/SFR in the ventrolateral region of the dermamyotome co-localized with that of a paired homeodomain containing gene, pax-3. Pax-3 is expressed in the entire dermamyotome and partial myotome at D9.5 of gestation, and is later expressed in migrating cells, as well as those cells that segregate into the dorsal and ventral portion of limb bud. Eventually in embryos at D11.5 of gestation, pax-3 expression overlaps with cells expressing *Myo* D and myogenin (Goulding et al., 1991; Bober et al., 1994; Goulding et al., 1994). A mouse homozygous mutant, Splotch, which has a deletion in the pax-3 gene, shows a dramatic loss of limb musculature corresponding with the loss of migrating pax-3 expressing cells in the limb (Epstein, et al., 1991; Franz et al., 1993, Bober et al., 1994; Goulding et al., 1994). Therefore, the pax-3 gene is the first molecular marker that has been identified for these migratory dermamyotome precursor cells of limb bud muscle (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). The observation that the *met*/HGF/SFR is only expressed in a few cells located to the ventrolateral region of the dermamyotome suggests that the *met*/HGF/SFR and pax-3 gene may somehow be related and the *met*/HGF/SFR may be a more precise marker for muscle precursor cells which migrate into the limb bud. Whether the pax-3 gene plays upstream function to the *met*/HGF/SFR and *Myo* D genes in these migrating muscle precursor cells remains to be tested. However, the migration phenomena

of several cell types in response to the treatment of HGF/SF in culture are well documented (Stoker et al., 1987, Rosen et al., 1989), and the kinase domain of *met*/HGF/SFR is sufficient to mediate these migratory signals (Weidner, et al., 1993; Komada and Kitamura, 1994; Zhu et al., 1994). Together, these observations suggest that one function of the *met*/HGF/SFR in these muscle precursor cells may be to stimulate these cells to migrate. However, I can not rule out the possibility that the *met*/HGF/SFR may play a role in the cell fate determination, proliferation and/or migration of myoblast stem cells destined to colonize the limbs and give rise to the different muscles of the limbs.

During development, especially the development of the somite and kidney, conversion of an epithelial to a mesenchymal cell phenotype frequently occurs prior to cell migration (Thiery et al., 1985; Duband et al., 1987; Hay, 1990). As mention above, when MDCK epithelial cells disperse on plastic in response to HGF/SF their morphology changes in a fashion that resembles an epithelial to mesenchymal transition (Stoker et al., 1987, Rosen et al., 1989). Moreover, the ectopic expression of HGF/SF and the *met*/HGF/SFR in NIH 3T3 cells leads to a partial conversion from mesenchymal to a more epithelial cell phenotype (Tsarfaty et al, 1994). Therefore, HGF/SF may be one of the factors that mediates the conversion between epithelial and mesenchymal cells. Somites are formed as the dorsal mesodermal cells aggregate and convert to an epithelial structure which further separates into an epithelial layer of dermamyotome, and mesodermal layers of myotome and scleratome. Some cells in the dermamyotome dissociate to form the mesodermal muscle precursors, as well as other cell types of the dermis (Emerson, 1993). The *met*/HGF/SFR is not expressed at stages when somites become a condensing mesenchymal epithelial structure, but it is expressed only in epithelial cells located in ventrolateral and dorsal-medial regions of dermamyotomes. This observation suggests that the *met*/HGF/SFR is not a general marker for epithelia in the somite but may play a

role in the conversion of these specific epithelial cells into a component that migrates to the limb bud.

Expression of the *met*/HGF/SFR is also detected in the endodermal layer of primitive gut and urinary tract in D9 and 10 embryos. At these stages the primitive gut and urinary tract are only simple tubules with a single layer of endodermal cells. During development, interactions of endodermal cells with adjacent mesodermal cells stimulate the proliferation and migration of endothelium, and possible conversion between epithelia and mesenchyme (Carlson, 1981, Casio et al., 1991). As a result, the primitive gut becomes divided anteroposteriorly into oesophagus, stomach, small intestine, cecum, large intestine and allantois. Along with these the primordial digestive glands (e.g. liver, pancreas) and respiratory system (e.g. thachea, lung) develop. Similarly the urinary tract also undergoes a developmental pattern in which the endothelium thickens, migrates, and follows a transition from mesenchyme to epithelia, and eventually becomes the pronephric tubule (which later degenerates), mesonephros (which later differentiates into tubulous structures including testis, uterus) and metanephros (definitive kidney). The observation that the *met*/HGF/SFR is expressed in the single layer of endothelium of primitive gut and urinary tract suggests that one function of the *met*/HGF/SFR may be in the proliferation of these epithelial stem cells. Expression of the *met*/HGF/SFR is also detected in the tubular epithelial cells of the developing (Soneberg et al., 1993) and adult organs (Chapter II) derived from the primitive gut, and urinary tract, indicating that the *met*/HGF/SFR may also be involved in other cellular events, such as migration and epithelial morphogenesis, involved in tissue architecture and homeostasis. In addition, HGF/SF is recognized as the first soluble mesenchymal factor isolated that can induce tubule formation of MDCK cells cultured in collagen matrix (Montaseno et al., 1991) and can stimulate formation of lumen-like structures from human epithelial carcinoma cell lines *in*

vitro (Tsarfaty et al., 1992). Moreover in collaboration we have shown that coculture of embryonic kidney with MDCK cells seeded in a collagen matrix induces the formation of branching morphogenesis of MDCK cells, which is inhibited following treatment with anti-HGF/SF antiserum (Santos et al., 1994). All together these data suggest that the stimulation of *met*/HGF/SFR may play a role in the proliferation and morphogenesis of these epithelial cells during development of the primitive gut and urinary tract.

In summary, from the studies of *met*/HGF/SFR expression presented in chapters II-IV it is clearly demonstrated that the *met*/HGF/SFR may be involved in epithelial cell proliferation, positioning and morphogenesis. In addition, the increase in the expression of *met*/HGF/SFR and its ligand during differentiation of P19 cells suggests that the stimulation of *met*/HGF/SFR may play a role in the differentiation of neuronal and mesodermal cells. The demonstration of the *met*/HGF/SFR expression in neuroepithelium and cells that correspond to the migrating muscle precursors, as well as in skeletal muscle myoblast cell line, C2C12 supports a role for the *met*/HGF/SFR in the migration and/or differentiation of neuronal cells and muscle cells during development.

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Chapter VI Contributions to original research

1. Localization of *met*/HGF/SFR expression in hepatocytes of the resting liver, granulosa cells of developing and mature follicles in the ovary, and in many tubular and surface epithelial cells in adult mouse tissues.

2. Demonstration of the expression of *met*/HGF/SFR and its ligand in undifferentiated P19 cells, and an increase in expression of *met*/HGF/SFR and its ligand as P19 cells differentiated into neuronal and mesodermal derivatives.

3. Demonstration of the presence of the *met*/HGF/SFR protein in neuronal cells derived from the RA-treated P19 cells and the expression of *met*/HGF/SFR mRNA in neuroepithelium of embryos.

4. Characterization of the expression of the *met*/HGF/SFR in cells that may correspond to those migrating muscle precursors which form limb bud muscles and the axial muscles of embryos.

5. Demonstration of the presence of *met*/HGF/SFR mRNA in the endodermal cells of primitive gut and urinal tract.