Met signaling mutants as tools for developmental studies

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ABSTRACT **The Met receptor is widely expressed in embryonic and adult epithelial tissues; its ligand (hepatocyte growth factor/scatter factor, HGF/SF) is expressed in the mesenchymal component of various organs. The generation of *hgf* and *met* null mice has revealed an essential role for this ligand-receptor pair in the development of the placenta, liver, and limb muscles. However the early lethality of the null mutants has precluded analysis of Met function in late development. To extend the possible observation period, we generated mutant *met* alleles of different severity. This was done by impairing the ability of the receptor to transduce the HGF/SF signal, *via* mutation of consensus sequences in the multifunctional docking site present in the C-terminal tail of the receptor. Mice expressing a Met mutant still active as a kinase, but unable to recruit its effectors, died in mid-gestation with the same phenotype as the *met* knockout, proving the importance of phosphotyrosine-SH2 interactions *in vivo*. Mice expressing a Met receptor with partial loss of signaling function survived until birth and revealed novel aspects of HGF/SF-Met function during muscle development.**

KEY WORDS: *Met signaling, Met in development, hypaxial muscle development.*

Introduction

Met tyrosine kinase is the receptor for HGF/SF (Bottaro *et al.*, 1991; Naldini *et al.*, 1991), a mesenchyme-derived pleiotropic growth factor, active on a variety of target cells. Among these are hepatocytes, melanocytes, keratinocytes, cells of the mammary, renal, gastric and biliary epithelium, as well as hematopoietic precursors, vascular endothelial cells, and neurons. The biological responses of cultured cells to HGF/SF range from survival and proliferation, to dissociation of epithelial sheets (scatter) and branching morphogenesis (for reviews see Gherardi and Stoker, 1991; Zarnegar and Michalopoulos, 1995; Boros and Miller, 1995; Matsumoto and Nakamura, 1996).

In vivo HGF/SF is a potent angiogenic factor (Bussolino *et al.*, 1992; Grant *et al.*, 1993) and is involved in regeneration of organs including liver, kidney and lung (Michalopoulos and De Frances, 1997; Matsumoto and Nakamura, 1997; Balkovetz and Lipschutz, 1999). Altered *met* expression and constitutive activation of the receptor have been described in human tumours and cell lines, suggesting a role for Met signaling in malignancy (Jeffers *et al.*, 1996). Recently, a direct genetic link between *MET* and human cancer has been established by identifying activating mutations in the Met kinase in hereditary and sporadic papillary kidney carcinoma (Schmidt *et al.*, 1997 and 1999).

In situ hybridization has revealed that transcripts of the ligand and the receptor are juxtaposed during embryogenesis, suggesting a paracrine mode of interaction and a possible role for HGF/SF-Met in epithelial-mesenchymal transitions occurring during development (Sonnenberg *et al.*, 1993 a and b). During organogenesis *hgf* is expressed at the highest level in placenta, liver and limb buds. The labyrinth layer of the placenta does not develop properly in *hgf* or *met* null mice, which, as a consequence, die in mid-gestation (Uehara *et al.*, 1995; Schmidt *et al.*, 1995). In these embryos the liver starts developing normally, but, beginning at E12.5, hepatocytes undergo massive apoptosis (Schmidt *et al.*, 1995). Finally, *c-met* null embryos lack limb and diaphragm muscles (Bladt *et al.*, 1995). This is due to the inability of mutant myoblast precursors to de-laminate from the lateral lip of the dermomyotome and initiate long-range migration in response to HGF/SF (Bladt *et al.*, 1995; Dietrich *et al.*, 1999). This phenotype recapitulates the 'scatter' effect originally seen in epithelial cells *in vitro*.

Given the embryonal lethality of the homozygous null for *hgf/sf* and *met* and considering that expression of the ligand and the receptor persists postnatally and in the adult, there are a number

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Abbreviations used in this paper: Met, Met protein; *met*, mouse gene; *MET*, human gene; HGF/SF, hepatocyte growth factor/scatter factor; etc.

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of questions left open on their physiological role. It remains to be determined, for instance, whether HGF/SF-Met are involved in morphogenesis of the mammary gland (as organ culture experiments seem to indicate, Yang *et al.*, 1995), or in liver and kidney regeneration, as suggested by a number of *in vivo* experiments. Lastly, nothing is known of the possible role for HGF/SF-Met in the adult brain, where they are co-expressed in the cortex, hippocampus and amygdala (Honda *et al.*, 1995).

Answers to some of these questions may come from tissue-specific or conditional knockouts for the Met receptor. As an alternative approach, we have generated mice carrying *met* alleles of different severity, taking advantage of the mechanism of signal transduction used by the Met receptor. Met is linked to all its effectors *via* ligand-induced phosphorylation of two critical tyrosine residues located in a multifunctional docking site (Y_{1349}VHVNATY$_{1356}$VNV) in the carboxy-terminal tail of the molecule (Ponzetto *et al.*, 1994). Effectors such as phosphatidylinositol 3-kinase (PI 3-Kinase), phospholipase Cγ (PLCγ) and Src can be directly recruited to the receptor *via* interaction with either of the two phosphotyrosines (Ponzetto *et al.*, 1993 and 1994). Grb2, the adapter for the Ras guanyl-nucleotide exchanger Sos, binds at high affinity only to the YVNV motif, given its requirement for an Asn in +2 (Songyang *et al.*, 1993). Downstream effectors can also be recruited indirectly *via* the Gab-1 adaptor, which is the major substrate for the Met kinase (Weidner *et al.*, 1996). Although in the yeast two hybrid system Gab-1 interacts directly with the bidentate docking site (Weidner *et al.*, 1996), *in vivo* it associates with Met mainly *via* Grb2 (Fixman *et al.*, 1997; Bardelli *et al.*, 1997). Thus the YVNV consensus is particularly critical for Met signaling, since it links the receptor with the Ras pathway and with the Gab-1 adaptor. Gab-1 is essential in at least some of the cellular responses to HGF (Fixman *et al.*, 1997; Bardelli *et al.*, 1997). It is, however, also phosphorylated in response to other growth factors and cytokines, among which are insulin and epidermal growth factor (EGF) (Holgado-Madruga *et al.*, 1996), nerve growth factor (NGF) (Holgado-Madruga *et al.*, 1997), and interleukin-6 (IL-6) (Takahashi-Tezuka, 1998).

Fig. 1. Met signaling mutants expressed by knock-in mice. *The recombinant Met receptor is a chimeric protein consisting of a murine extracellular domain (red) fused to a human transmembrane and cytoplasmic domain (black). met$^{Wt/Wt}$, chimeric protein with wild-tipe multifunctional docking site; met$^{D/D}$, chimeric protein without docking site; met$^{Grb2/Grb2}$, chimeric protein with disrupted Grb2 binding site.*

Indeed, the phenotype of Gab-1 null embryos has features which partially overlap with those observed not only in embryos lacking HGF/SF, but also platelet growth factor (PDGF), EGF and leukemia inhibiting factor (LIF), confirming that this molecule is a signal amplifier common to a broad range of receptors (Itoh *et al.*, 2000).

We and others have shown that Y→F mutation of the two tyrosines of the multifunctional docking site (Met^{Double}, thereafter indicated as Met^D) abrogates receptor function in transfected cells (Ponzetto *et al.*, 1994; Zhu *et al.*, 1994; Weidner *et al.*, 1995; Fixman *et al.*, 1995). Conversely, N→H mutation in the YVNV consensus (resulting in the loss of the Grb2 binding site, Met^{Grb2}) interferes with some (but not all) Met-mediated events. The signal conveyed by the Met^{Grb2} mutant is in fact permissive for motility (Ponzetto *et al.*, 1996), but inadequate to promote branching tubulogenesis in MDCK cells (Fournier *et al.*, 1996), or for Tpr-Met-mediated transformation (Ponzetto *et al.*, 1996).

Having analysed the consequences of lowering Met signaling, we next attempted to correlate Met-mediated biological effects with the activation of specific pathways. This is more difficult for Met, which relies on a bidentate docking site of mixed specificity, than for other receptors (such as the PDGFR) possessing multiple docking sites of high specificity. These in fact can be mutated one by one to evaluate the consequences of the loss of single pathways (Fantl *et al.*, 1992). In the case of Met we modified the consensus sequences of the multifunctional docking site to enhance direct coupling with Grb2 (Met^{2xGrb2}) or with PI 3-kinase (Met^{2xPI3K}). Both mutants resulted in a partial loss of biological function when tested in transfected cells (Giordano *et al.*, 1997; Bardelli *et al.*, 1999), confirming that the full spectrum of physiological effects of HGF requires activation of a delicate balance of downstream effectors.

The Met signaling mutants described above have all been transferred in the mouse by homologous recombination. This review summarises the results obtained with the first set of mutations, aimed at lowering Met signaling either severely (Met^D) or weakly (Met^{Grb2}) (Maina *et al.*, 1996; see Fig. 1). The mice generated by introduction of the Met^{2xGrb} and Met^{2xPI3K} mutations will be described elsewhere (Maina *et al.*, submitted).

Results

Uncoupling effectors from the Met receptor in vivo *results in a mid-gestation lethal phenotype, similar to that of the met null*

We sought to determine the importance of the bidentate docking site Y_{1349}VHVNATY$_{1356}$VNV in Met signaling *in vivo* by introducing Y→F mutations of tyrosine 1349 and 1356 in the mouse genome. This was done by knocking-in the *met* locus a mutated human cDNA fragment coding for the transmembrane and cytoplasmic domain of the receptor (Maina *et al.*, 1996). The resulting recombinant Met protein (*met^D*) consisted of a murine extracellular domain fused to a human transmembrane and cytoplasmic domain (Fig. 1). As a control we also knocked-in a wild type human *MET* cDNA (*met^{WT}*). Homozygous *met$^{WT/WT}$* mice were normal, while homozygous *met$^{D/D}$* embryos died *in utero* in mid-gestation, with the same timing and phenotype as the *met* knockout (Maina *et al.*, 1996; Birchmeier and Gherardi, 1998). In particular, from E 12.5 on, in *met$^{D/D}$* embryos the development of the placenta was impaired and the size of the liver was reduced. Tunel staining revealed numerous apoptotic nuclei in mutant hepatocytes. Furthermore, these embryos lacked a subset of skeletal muscles
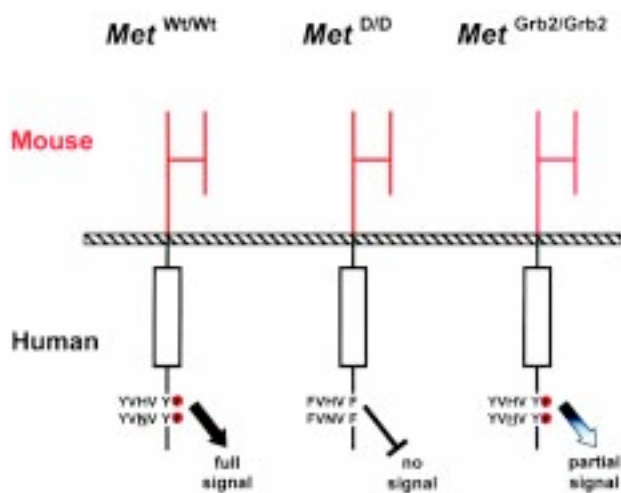
(hypaxial muscles of the limbs, diaphragm and tip of the tongue) which all derive from migratory precursors (Dietrich *et al.* 1999). Deep muscles of the back and intercostals (epaxial), which develop *in situ* (Denetclaw *et al.*, 1997), were normal.

The description of the skeletal muscle defects of the *met*^{D/D} embryos was further refined by crossing these mice with transgenics expressing the *lacZ* gene under the myosin light chain promoter (Kelly *et al.*, 1997). Staining for β-galactosidase activity revealed that, in addition to the absence of appendicular muscles, *met*^{D/D} embryos also lacked superficial body wall muscles (Prunotto *et al.*, in preparation). The latter derive, like appendicular muscles, from myoblast precursors found in the ventro-lateral lip of the dermomyotome. However, in the flank these precursors do not disperse, but remain epithelially arranged while the dermomyotome elongates as a continuous sheet of cells (Dietrich *et al.*, 1998); proliferation, rather than active migration, appears to be the driving force of dermomyotomal elongation. This defect suggests that during hypaxial muscle development HGF/SF-Met may have a more complex role than simply controlling emigration of myoblast precursors.

Finally, *met*^{D/D} mutants were useful to better define the emerging neurogenic role for HGF/SF and Met (Maina and Klein, 1999). *In vitro* studies suggested that HGF/SF could act as a chemoattractant and a survival factor for a subpopulation of embryonic motor neu-

Fig. 2. Limb muscle defects of the *met*^{Grb2/Grb2} homozygous mutant. *Homozygous mutant neonates (B) display abnormal position of forelimbs compared with control littermates (A). Histological analysis of sections distal to the knee of the hind limb from (C) control and (D) mutant met^{Grb2/Grb2} neonates. Transverse paraffin sections were stained with haematoxylin and eosin. T, tibia; F, fibula. Individual muscles are indicated by numbers: 1, extensor digitorum longus; 2, soleus; 3, gastrocnemius. (F) met^{Grb2/Grb2} survivor at age two weeks; the animal retains the hyperflexed forelimb phenotype and is smaller than (E) the control.*

rons (Ebens *et al.*, 1996; Yamamoto *et al.*, 1997; Wong *et al.*, 1997). The analysis of *met*^{D/D} embryos indeed showed defects at the level of limb motor innervation (shortening of motor nerves and reduction of branching, Maina *et al.*, 1997). These studies also revealed a role for HGF/SF-Met in survival and axonal outgrowth of sensory (Maina *et al.*, 1997) and sympathetic neurons (Maina *et al.*, 1998), and in differentiation of their precursors. The network of sensory fibres innervating the skin of the limbs and the trunk was found to be greatly reduced in *met*^{D/D} embryos. Furthermore HGF/SF dramatically enhanced NGF-induced neurite outgrowth from cultured dorsal root ganglia (DRG) explanted from control mice, while DRGs from *met*^{D/D} mutants did not respond. Analysis of DRG sections during the period of Met-dependent neurite outgrowth (E12.5) revealed a consistent increase in cell death in the mutant embryos (Maina *et al.*, 1997). Lastly, cultures of superior cervical ganglia from *met*^{D/D} embryos were used to show that HGF enhances the survival and differentiation of sympathetic neuroblasts and cooperates with NGF to enhance the axonal and neurite outgrowth of sympathetic neurons (Maina *et al.*, 1998).

Lowering the signaling threshold of the Met receptor *in vivo* results in a hypomorphic mutant which develops to birth

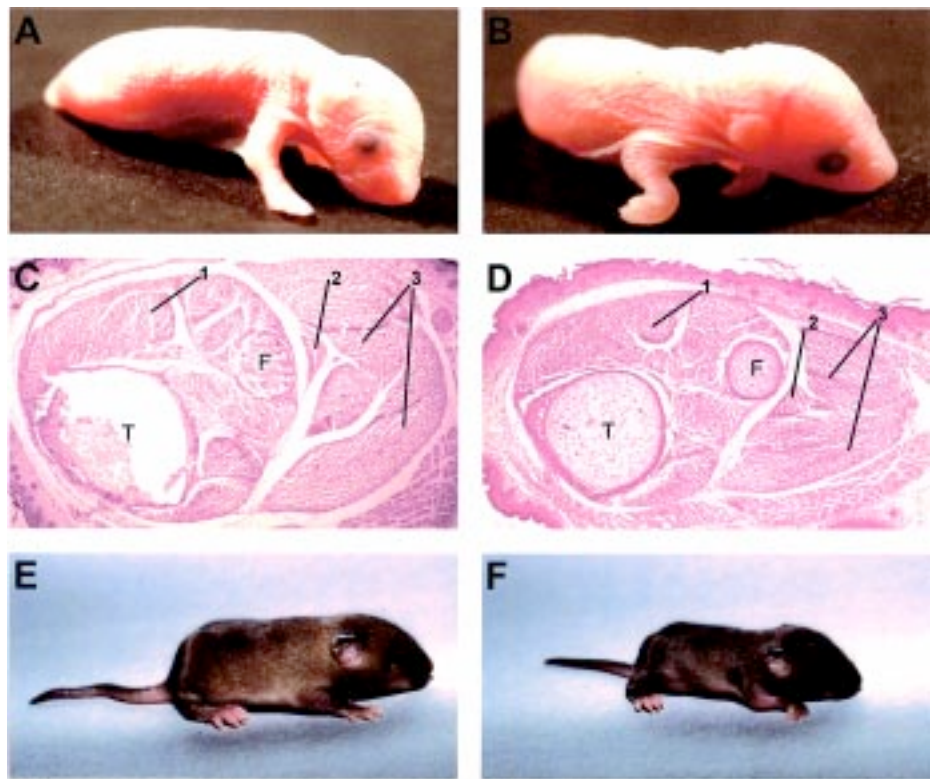To obtain a hypomorphic mutant for the Met receptor we knocked-in the *met* gene a mutated cDNA carrying a N→H muta-

tion in the YVNV consensus of the bidentate docking site, resulting in the loss of Grb2 binding (*met*^{Grb2}, Maina *et al.*, 1996). This mutant in transfected cells was permissive for motility but not for tubulogenesis or transformation (Ponzetto *et al.*, 1996; Fournier *et al.*, 1996). Homozygous *met*^{Grb2/Grb2} mice were born alive. The placenta appeared to be morphologically normal, indicating that the signaling output of the *met*^{Grb2} mutant is sufficient to rescue placental development. The liver and motor and sensory innervation also appeared normal in *met*^{Grb2/Grb2} mice (Table 1; Maina *et al.*, 1996 and 1997).

On the other hand, *met*^{Grb2/Grb2} mice had hyperflexed forelimbs, weak hind limbs, and showed respiratory distress, due to a significant reduction of appendicular and diaphragm muscles (panel A and B of Fig. 2 and Maina *et al.*, 1996). Forelimbs seemed to be more severely affected than hind limbs. In forelimbs, extensors (derived from precursors of the dorsal pre-muscle mass) were more severely reduced than flexors (Maina *et al.*, 1996), thus explaining the hyperflexed forelimb phenotype. Panel C and D of Fig. 2 show sections of the hind limb of a control mouse and of a *met*^{Grb2/Grb2} mutant. In this case, flexor muscles (including the *gastrocnemius*), derived from the ventral pre-muscle mass, seemed to be the most affected. It should be noted that in these mice migratory precursors of appendicular muscles appeared to delaminate properly from the somite, and that at least the initial
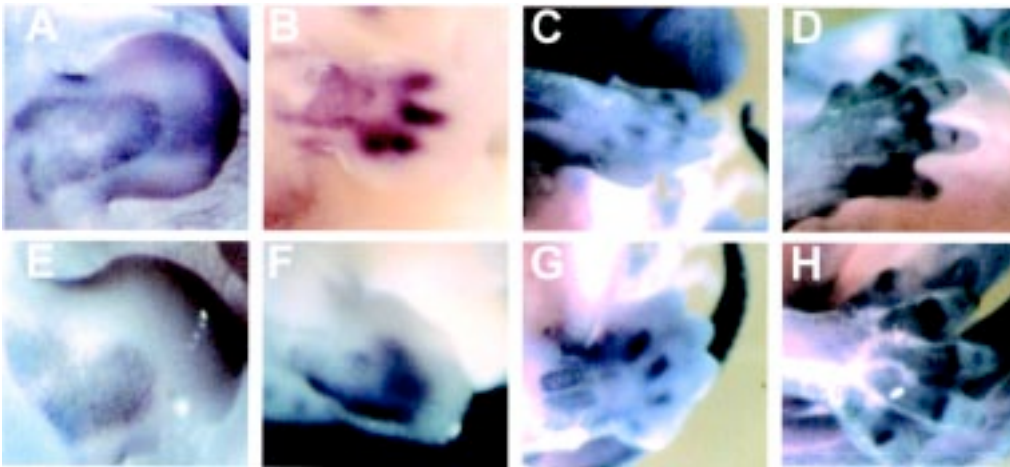
Fig. 3. Expression of *met* during limb development. *Whole-mount in situ hybridization with a digoxigenin labelled met probe. A-D, fore limb; E-H, hind limb.* **(A** *and* **E)** *At E11.5, met is highly expressed in proliferating embryonal myoblasts.* **(B** *and* **F)** *At E12.5, met expression is downregulated proximally and persists distally along the digits, corresponding to pre-cartilaginous condensations.* **(C** *and* **G)** *At E13.5, met is re-expressed in the developing muscles, in concomitance with the proliferation of fetal myoblasts.* **(D** *and* **H)** *At E14.5, met expression is mainly localised in the joints region.*

stages of their migration into the limb bud did not seem to be compromised (Maina *et al.*, 1996).

The majority of the *met*$^{Grb2/Grb2}$ pups died in the first hours after birth most likely because of their inability to breathe properly, due to their weak diaphragm (Maina *et al.*, 1996). Occasionally a few *met*$^{Grb2/Grb2}$ mutants survived into adulthood. During the first few postnatal weeks they did not increase in size like the controls, retained the hyperflexed phenotype in their forelimbs, and showed a reduction in total muscle mass (see panel E and F of Fig. 2). However with time these animals did recover in body size, muscle mass and strength.

Surprisingly, histological analysis of muscle sections of *met*$^{Grb2/Grb2}$ new-borns also showed abnormal morphology in intercostal and deep back (epaxial) muscles. Epaxial muscles derive from non-migratory precursors originating in the dorsal-medial lip of the dermomyotome, a site which, like the ventro-lateral lip of the somite, is positive for Met expression (Yang *et al.*, 1996; Denetclaw *et al.*, 1997). Up to mid-gestation, however, Met signaling must be redundant for the development of these muscles, since they are unaffected in the more severe *met* null and *met*$^{D/D}$ embryos. The appearance of this defect in later stages of myogenesis suggests that Met may be required to mediate proliferation of fetal myoblasts from which secondary fibres are formed. Indeed bromo-deoxyuridine incorporation into myogenic nuclei at E17.5 was found to be reduced in intercostal muscles of *met*$^{Grb2/Grb2}$ embryos, suggesting that HGF/SF may contribute to trigger proliferation of all fetal myoblasts *in vivo* (Maina *et al.*, 1996).

The temporal pattern of *met* expression in fetal limb muscles supports this possibility. *Met* is highly expressed up to day E11.5 in the proliferating embryonal myoblasts forming the dorsal and ventral pre-muscle masses (see panels A and E in Fig. 3). At E12.5 *met* is downregulated in the differentiated primary fibres in the limbs. Its expression is maintained only distally along the digits, presumably in correspondence of pre-cartilaginous condensations (panels B and F in Fig. 3). At day 13.5, in concomitance with resumption of proliferation of fetal myoblasts, *met* is re-expressed in the developing muscles (panel C and G in Fig. 3). Interestingly, at E14.5 the highest level of *met* expression is in the region of the future joints, where expression of HGF has also been demonstrated (Takebayashi *et al.*, 1995). Thus the HGF/SF-Met pair could play a role in chondrocyte development as well.

HGF/SF enhances DNA synthesis in cultured epaxial fetal myoblasts

To verify the effect of HGF/SF on epaxial fetal myoblasts, deep back muscles were removed from E13.5 embryos, digested with collagenase and cultured. Addition of HGF to these cultures resulted in a 24% increase in BrdU incorporation in myogenic (desmin positive) cells relative to controls (Fig. 4). A similar proliferative response was elicited by basic fibroblast growth factor (bFGF). This *in vitro* result supports a possible role for HGF/SF on fetal myoblast proliferation *in vivo*.

HGF/SF promotes proliferation and delays differentiation of satellite cells in culture

A proliferative role for HGF/SF has been demonstrated for satellite cells, myoblasts which lie quiescent under the basal lamina of adult muscle fibres. Met is expressed in quiescent satellite cells (Cornelison and Wold, 1997), and HGF/SF is capable of activating satellite cells *in vitro* (Allen *et al.*, 1995; Gal-Levi *et al.*, 1997; Tatsumi *et al.*, 1998). This has suggested a potential role for HGF/SF in the early phase of muscle regeneration, when satellite cells must re-enter the cell cycle. Indeed, in the rat, transcription of HGF/SF increases during the first few days after muscle injury (Jennische *et al.*, 1993). To verify the proliferative effect of HGF/SF on satellite cells, we prepared cultures from single muscle fibre explants obtained from adult mice (panel A of Fig. 5; Bischoff, 1986). Such preparations result in a higher yield and purity of satellite cells than those obtained starting from minced muscle. Single fibres were maintained in enriched growth medium until the

TABLE 1.

PHENOTYPE OF EMBRYOS HOMOZYGOUS FOR THE INDICATED *MET* SIGNALING MUTANTS

	Placenta	Liver (Limb and body wall)	Muscle	Motor Nerves	Sensory nerves
met$^{Wt/Wt}$	+	+	+	+	+
met$^{D/D}$	-	-	-	-	-
met$^{Grb2/Grb2}$	+	+	±	+	+

Fig. 4. HGF/SF stimulates BrdU incorpora-tion in epaxial fetal myoblasts in culture. *Deep back muscle was taken from E13.5 em-bryos of mixed genetic background, digested with collagenase, and plated for 5 h in high serum medium, after a 10 min pre-plating to remove part of the fibroblasts. The medium was then switched to low serum with or without growth factor for 8 h before the addition of BrdU. Cells were incubated with BrdU overnight and*

then fixed and stained using anti-desmin (Cy-3, red) and anti-BrdU (FITC, green) antibodies to discriminate proliferating myogenic from non-myogenic cells. **(A)** *BrdU incorporation in low serum condition shows few BrdU/desmin positive cells.* **(B)** *Addition of 50 U/ml of recombinant HGF/SF to low serum medium resulted in a 24 % increase in the number of double-labelled cells relative to (A).* **(C)** *A similar proliferative response was elicited using 20 ng/ml of recombinant bFGF.*
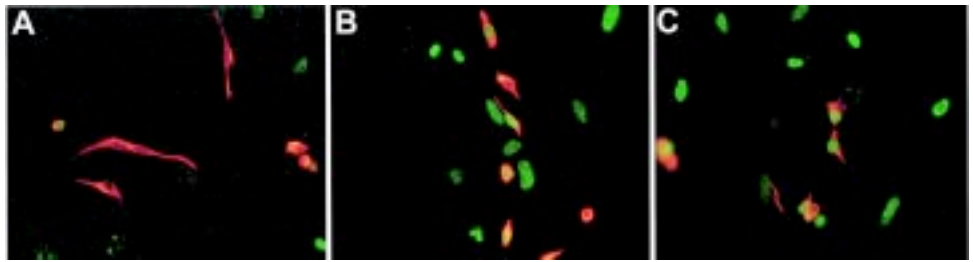
few associated satellite cells migrated out of the basal lamina (panel B Fig. 5). On the third day, cultures were switched to differentiation medium with or without addition of recombinant HGF/SF. Fig. 5 shows the appearance of these cultures five days after the switch. Satellite cells rapidly differentiated into small myotubes in differentiation medium (panel C). Addition of HGF resulted in a delay in differentiation and a significant increase of small round mono-nucleate satellite cells (panel D). A similar dual effect of HGF/SF has also been observed in cultures of chicken satellite cells (Gal-Levi *et al.*, 1997; Miller *et al.*, 2000).

HGF/SF induces cyclin D1 expression and nuclear transloca-tion of cdk4 in C2C12 myogenic cells

To understand the basis for the proliferative response of myoblasts to HGF/SF we studied its effects on cyclin D1 and cdk4, which are involved in the control of cell cycle progression and in inhibition of myogenic differentiation. For these studies we used the C2C12 myoblast cell line, where it has been shown that myogenic differen-tiation and MyoD expression can be inhibited by transfection of a constitutively active Met kinase (Anastasi *et al.*, 1997).

Panel A in Fig. 6 shows that cyclin D1 is undetectable in the nuclei of starved C2C12 cells. A six hours treatment with HGF/SF induced the appearance of cyclin D1 in the majority of the myogenic nuclei (panel B). Concomitant with this increase there was also nuclear translocation of cdk4 (panels C and D). Modulation of cyclin D1 levels and nuclear translocation of the cyclin D1-cdk4 complex has been described as a major mechanism by which mitogens inhibit myo-genic differentiation (Zhang *et al.*, 1999 a and b).

Discussion

The approach that we have taken to modify Met function *in vivo* (interfering with Met signaling by mutation of SH2 docking sites) was based on knowledge of the mechanism adopted by this and other tyrosine kinase receptors to carry out signal transduction. Tyrosine kinase receptors recruit cytoplasmic effectors via short recognition motifs, composed of phosphotyrosine followed by three to five downstream residues, which are generated by autophosphorylation upon ligand-dependent receptor activation. Effectors associate with the receptor with their own SH2 domains or by forming a complex with SH2-containing adaptors. Phosphotyrosine-SH2 interactions are usually quite specific, thus the sequence of the SH2 docking sites on a given receptor dictates which SH2-containing targets will associate with the receptor (for a review see Pawson and Scott, 1997).

Met, the receptor for HGF/SF and the closely related members of its family Ron and Sea, represent an unusual case. Rather than possessing numerous specific docking sites, they rely on two closely spaced phosphotyrosines in their carboxy-terminal tail to convey the bulk of their signaling activity (Ponzetto *et al.*, 1994; Iwama *et al.*, 1996; Park and Hayman, 1999). These tyrosine residues are part of a bidentate 'multifunctional' docking site (Y_{1349}VHVNATY$_{1356}$VNV in Met), which can bind a number of SH2-containing signaling proteins. In all three members of the Met family the second phosphotyrosine is embedded in an optimal motif for the Grb2 adaptor. Grb2 links the Met receptor with two critical effectors, the Ras guanyl-nucleotide exchanger Sos and the Gab-1 protein (Ponzetto *et al.*, 1994; Fixman *et al.*, 1997; Bardelli *et al.*, 1997). Gab-1 is a signal amplifier common to other tyrosine kinase receptors (Holgado-Madruga *et al.*, 1996). It con-tains a pleckstrin domain and a number of potential phosphoryla-
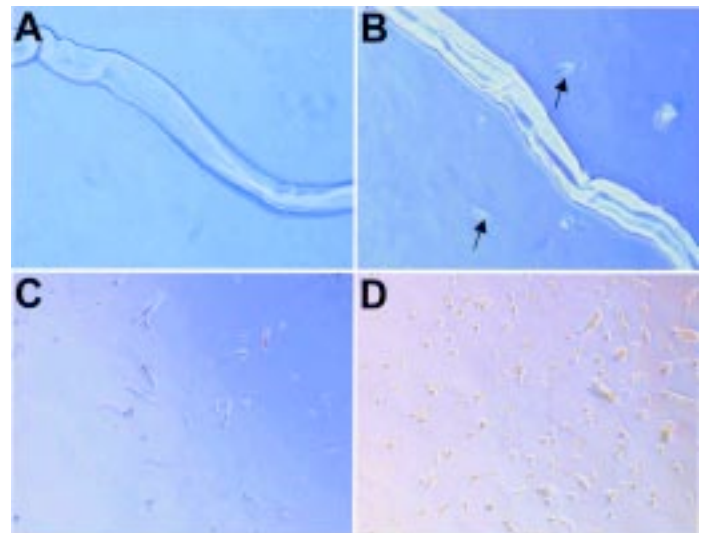

Fig. 5. HGF/SF promotes proliferation and delays differentiation of satellite cells in culture. *High purity satellite cell cultures were obtained using single muscle fibre explants from adult mice.* **(A)** *Isolated single muscle fibres were maintained in enriched growth medium until* **(B)** *the few associated satellite cells (black arrows) migrated out of the basal lamina (two to three days). Cultures were then switched to low serum and photographed five days later.* **(C)** *Lowering the serum concentration resulted in rapid differentiation of satellite cells into small myotubes.* **(D)** *The addition of 50 U/ml of recombinant HGF/SF resulted in fewer myotubes and in an increase of small round mononucleate satellite cells.*
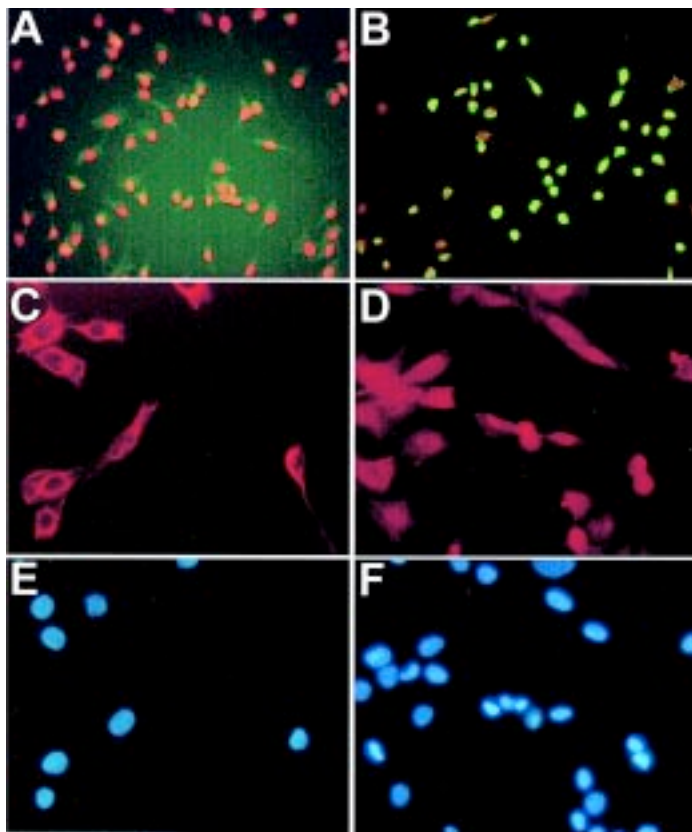
Fig. 6. HGF/SF induces cyclin D1 expression and cdk4 nuclear translocation in C2C12 myogenic cells. *C2C12 myoblasts were starved at low serum for 14 h and stained using antibodies specific for* **(A,B)** *cyclin D1 and* **(C,D)** *cdk4.* **(A)** *Cyclin D1 is undetectable in the nuclei, stained in Red by DAPI.* **(B)** *A 6 h treatment with 50U/ml of recombinant HGF/SF induced cyclin D1 expression (FITC, green).* **(C, D)** *The same treatment of C2C12 myogenic cells resulted in the nuclear translocation of cdk4 (Cy-3 red).* **(E,F)** *DAPI staining.*

tion sites, some of which are optimal binding sites for PI 3-kinase, PLC-γ, and the tyrosine phosphatase SHP2. Gab-1 is critical for mediating HGF-induced branching tubulogenesis in MDCK cells (Weidner *et al.*, 1996; Maroun *et al.*, 1999).

We tested *in vivo* the effects of abrogating recruitment of SH2-containing effectors to the Met receptor without interfering with its tyrosine kinase activity, by converting to phenylalanine the two tyrosine residues of the Met multifunctional docking site (Met^D) *via* homologous recombination. The resulting phenotype showed the same defects as the *met* null (placenta, liver, muscle, and motor innervation), confirming for the first time in mice the importance of phosphotyrosine-SH2 interactions in tyrosine kinase receptor signaling (Ponzetto, 1998).

In particular, from E13.5 on, the placenta of *met*$^{D/D}$ embryos was paler and smaller than controls. Analysis of the placenta in *hgf* and *met* null mice revealed a reduction in the trophoblast cells of the labyrinth layer (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). Interestingly, the same defect has recently been described in Gab-1 null mice (Itoh *et al.*, 2000), suggesting that HGF/SF-Met signaling through Gab-1 is critical for development of these cells. *met*$^{D/D}$ mutants also have an apoptotic liver and lack muscles of the limbs, diaphragm and tip of the tongue, as observed in the *met* null (Bladt

et al., 1995). In these embryos, muscle precursors at limb level in the lateral dermomyotomal lips do not undergo de-epithelialization and do not enter the limb bud, confirming that Met signaling in myogenic precursors is essential for the initial steps of long-range migration.

Interestingly, however, the Met ligand is expressed along the entire route followed by migratory cells and in their target sites, suggesting that HGF/SF may guide muscle precursors toward their final destination (Dietrich *et al.*, 1999). Experiments done by applying beads soaked with HGF/SF to the flank of chick embryos to verify its chemotactic effect were inconclusive (Heymann *et al.*, 1996; Brand-Saberi *et al.*, 1996). The strongest indication of a true chemotactic role for HGF/SF comes from the phenotype of transgenics expressing HGF/SF ectopically. In these mice there is aberrant development of muscles around the spinal cord, implying misrouting of migratory myoblasts toward the ectopic source of HGF/SF (Takayama *et al.*, 1996). However, HGF/SF in these animals is probably at far higher than physiological levels. The prolonged requirement for active Met signaling during long range migration may fulfil additional requirements of migrating cells, such as the need to maintain the undifferentiated state (Scaal *et al.*, 1999) or to sustain their proliferation.

The additional Met mutant that we tested *in vivo* was obtained by modifying the consensus sequence of the second of the two tyrosines of the multifunctional docking site to uncouple the Grb2 adaptor (Y_{1356}VNV→Y_{1356}VHV, Met^{Grb2}). This mutation, besides impairing activation of the Ras pathway (Besser *et al.*, 1997) has the effect of lowering overall Met signaling, since Grb2 is responsible for linking the Met receptor not only to Sos but also to the signal amplifier Gab-1. In transfected MDCK cells, over-expression of Gab-1 can rescue the branching tubulogenesis defect of this mutant, but not of the Met^D mutant. The Met^{Grb2} mutant may thus retain some residual binding capacity for Gab-1, since the latter may directly interact with the mutated bidentate docking site (Maroun *et al.*, 1999).

The phenotype of the *met*$^{Grb2/Grb2}$ mutants was milder than that of the *met*$^{D/D}$ mice. The residual signaling ability of Met^{Grb2} seems to be sufficient for normal development of placenta, liver, and motor and sensory innervation. The tissue most sensitive to the lowered Met signaling threshold appeared to be skeletal muscle. *met*$^{Grb2/Grb2}$ mice showed severe reduction of appendicular and diaphragm muscles, deriving from migratory precursors. Muscle cell progenitors, however, did not seem to be impaired in their ability to delaminate from the somite and to start their migration. Similarly, the timing of their differentiation did not seem to be altered. A possible explanation for the partial defect observed could be failure of myogenic precursors to survive or to proliferate properly, while on route toward their site of destination. This would again imply a more complex role for HGF/SF-Met on embryonal myoblasts than that of mediator of somite de-epithelialization and chemotaxis.

There are several other hints that support this hypothesis. First of all by crossing the *met*^{D} mutant with mice expressing *lacZ* under the myosin light chain promoter (Kelly *et al.*, 1997) we observed that *met*$^{D/D}$ embryos also lack superficial body wall muscles, besides muscles of the limbs. The former belong to the hypaxial group deriving from the ventro-lateral dermomyotome, but their precursors do not undergo active migration. These muscles are laid down along the flanks of the embryo as the lateral lips of the

dermomyotome elongate by continuous proliferation. Thus the role of Met in their development must be distinct from that of mediating somite delamination and/or long range migration.

Furthermore, *met* is expressed from early embryonic stages in the dorsal lip of the dermomyotome where precursors of epaxial muscles originate (Yang *et al.*, 1996). In *met*$^{D/D}$ and in *met*$^{Grb2/Grb2}$ mutants epaxial muscles are normal up to mid-gestation, indicating that Met signaling is redundant during primary myogenesis. However, histological analysis of these muscles in *met*$^{Grb2/Grb2}$ mice at birth reveals a reduction in secondary fibres. Met may thus contribute to the development of these muscles at the onset of secondary myogenesis, when fetal myoblasts re-enter the cell cycle and resume proliferation, after temporary quiescence. Indeed the proliferative capacity of fetal myogenic cells appears to be reduced in *met*$^{Grb2/Grb2}$ embryos, as indicated by lower BrdU incorporation *in vivo*. In support of this hypothesis, we have now shown that cultures of epaxial myoblasts obtained from E13.5 embryos respond to HGF/SF by an increase in BrdU incorporation. Nevertheless, the role of HGF/SF in fetal myoblast proliferation remains controversial. In fact Dietrich *et al.* (1999), who recently obtained perinatal *met* null fetuses after tetraploid rescue of the placenta, did not find any defect in trunk muscles.

On the other hand several reports describe a proliferative effect of HGF/SF on satellite cells (Allen *et al.*, 1995; Gal-Levi *et al.*, 1997; Tatsumi *et al.*, 1998 and this review). This, together with the observed increased level of expression of HGF/SF in the first few days after muscle injury (Jennische *et al.*, 1993) has suggested that HGF/SF may trigger muscle regeneration *in vivo*. Proof of involvement of HGF/SF-Met in regeneration (of muscle or of any tissue) awaits a viable loss-of-function model. This will most likely be a conditional knockout.

We have recently obtained a viable *met*$^{Grb2/Grb2}$ mutant making use of a modified knock-in targeting vector in which the *neo* cassette (see Maina *et al.*, 1996) was flanked by *loxP* sites. Neo sequences are known to de-stabilise transcripts, and for this reason it is desirable to remove them from recombinant loci. This was done by crossing the new mutant with the Cre *Deleter* (Schwenk *et al.*, 1995). The resulting *met*$^{Grb2/Grb2neo-}$ mutants are viable and apparently normal. This suggests that the previous *met*$^{Grb2/Grb2}$ mice represent a 'sensitised' model, where the effects of the partial loss of function mutation (Y$_{1356}$VNV→Y$_{1356}$VHV) and of a decreased level of *met* transcript (and protein) add up to produce the observed phenotype. Viable *met*$^{Grb2/Grb2neo-}$ mutants, although apparently free of developmental defects, may still prove useful to evaluate the role of HGF/SF-Met in tissue and organ regeneration, and their function in adult tissues such as brain, where a higher threshold of Met signaling may be required.

Since our first report on the generation of mice expressing Met signaling mutants by homologous recombination (Maina *et al.*, 1996), the same approach has been applied to a number of receptors. Y→F mutation of the tyrosine residue leading to activation of PLCγ in the Fibroblast Growth Factor Receptor 1 (FGFR1) surprisingly revealed that this docking site mediates a negative rather than a positive signal (Partanen *et al.*, 1998). On the other hand, mutation of the Shc binding site in *trk*B unveiled a difference in the signaling output triggered by the two neurotrophins NT4 and BDNF, with the former being less potent than the latter in eliciting the MAP kinase response (Minichiello *et al.*, 1998). Mutation of the two PI 3-kinase docking sites in the PDGF-β receptor did not interfere with development, but cells derived from the mutants were less chemotactic and lost the ability to contract collagen gels in response to PDGF (Heuchel *et al.*, 1999). Furthermore, injection of a mast cell de-granulating agent in these mice led to a decrease in interstitial fluid pressure, resulting in edema. Thus the signaling mutant revealed a PI 3-kinase-mediated role for the PDGF-β receptor in interstitial fluid homeostasis. Loss of the PI 3-kinase docking site in the Kit receptor induced a gender and tissue-specific defect (Blume-Jensen *et al.*, 2000). Although there were no hematopoietic or pigmentation defects in homozygous mutant mice, males were sterile, due to a block in spermatogenesis. Lastly, knock-in mice expressing signaling mutants of gp130 (a common subunit for the interleukin-6 family of cytokine receptors) were born apparently normal but with immunological defects in the case of SHP2 signal-deficient mice, and were perinatal lethal (as the gp130 null) in the case of the STAT3 signal-deficient mice (Ohtani *et al.*, 2000).

In conclusion, signaling mutants represent a valuable tool to generate a spectrum of alleles which may help to unravel physiological roles for the corresponding receptors during development and in the adult, and may allow dissection of pathways involved in the biological response to the ligand.

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