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 (Sonnencamp 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

Abbreviations used in this paper: Met, Met protein; met, mouse gene; MET, human gene; HGF/SF, hepatocyte growth factor/scatter factor; etc.
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 experi-
ments 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, hippocam-
pus 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 alterna-
tive 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 (Y1349VHVNATY1356VNV) in
the carboxy-terminal tail of the molecule (Ponzetto et al., 1994).
Effectors such as phosphatidylinositol 3-kinase (PI 3-Kinase), phos-
photidylinositol 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).

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 (MetDouble, thereafter
indicated as MetD), 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, MetGrb2
interferes with some (but not all) Met-mediated events. The signal
conveyed by the MetGrb2 mutant is in fact permissive for motility
(Ponzetto et al., 1996), but inadequate to promote branching
of 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
(Fanit et al., 1992). In the case of Met we modified the consensus
sequences of the multifunctional docking site to enhance direct
coupling with Grb2 (Met2xGrb2) or with PI 3-kinase (Met2xPI3K).
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 muta-
tions, aimed at lowering Met signaling either severely (MetD) or
weakly (MetGrb2) (Maina et al., 1996, see Fig. 1). The mice
generated by introduction of the MetGrb2 and Met2xPI3K 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 Y1349VHVNATY1356VNV 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 (metD) 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 (metWT). Homozygous metWT/WT mice were normal, while
homozygous metD/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 metD 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. Fur-
thermore, these embryos lacked a subset of skeletal muscles
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 epithelioidly 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 neurons (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 mutation 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

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

The majority of the met<sup>Grb2/Grb2</sup> 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<sup>D/D</sup> 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<sup>Grb2/Grb2</sup> 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 the proliferation of fetal myoblasts, met expression is mainly localized in the joints region.

**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., 1995; 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

| PHENOTYPE OF EMBRYOS HOMOZYGOUS FOR THE INDICATED MET SIGNALING MUTANTS |
|---------------------|---------|--------|-------|--------|--------|
|                     | Placenta | Liver (Limb and body wall) | Muscle | Motor Nerves | Sensory nerves |
| met<sup>Wt/Wt</sup> | +       | +      | +     | +      | +      |
| met<sup>D/D</sup>   | -       | -      | -     | -      | -      |
| met<sup>Grb2/Grb2</sup> | +   | +      | +     | +      | +      |
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 translocation 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 differentiation and MyoD expression can be inhibited by transfection of a myoblast cell line, where it has been shown that myogenic differentiation 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 myogenic 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).
In particular, from E13.5 on, the placenta of metD/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. metD/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 transgensics 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 fulfill 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 uncleave the Grb2 adaptor (Y1356→VNV→Y1356→VHV, MetGrb2). 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 MetD mutant. The MetGrb2 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 metGrb2/Grb2 mutants was milder than that of the metD/D mice. The residual signaling ability of MetGrb2 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. metGrb2/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 metD mutant with mice expressing lacZ under the myosin light chain promoter (Kelly et al., 1997) we observed that metD/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 mef<sup>1</sup>D and in mef<sup>Grb2/Grb2</sup> 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 mef<sup>Grb2/Grb2</sup> 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 mef<sup>Grb2/Grb2</sup> embryos, as indicated by lower BrdU incorporation in myoblast cultures of epaxial myoblasts obtained from E13.5 embryos responding 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 mef<sup>Grb2/Grb2</sup> 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 mef<sup>Grb2/Grb2 neo</sup> mutants are viable and apparently normal. This suggests that the previous mef<sup>Grb2/Grb2</sup> mice represent a ‘sensitised’ model, where the effects of the partial loss of function mutation (Y<sub>1356</sub>VNV→Y<sub>1356</sub>VHV) and of a decreased level of met transcript (and protein) add up to produce the observed phenotype. Viable met<sup>Grb2/Grb2 neo</sup> 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 PLC<sub>γ</sub> 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 trkB 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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