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Activation of Met Tyrosine Kinase by Hepatocyte Growth Factor Is Essential for Internal Organogenesis in Xenopus Embryo

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Hepatocyte growth factor (HGF) specifically activates Met tyrosine kinase receptor, leading to mitogenic, motogenic, and morphogenic responses in a wide variety of cells. To know a role of HGF in Xenopus embryogenesis, loss-of-function mutation was introduced by dominant expression of truncated tyrosine kinase-negative Met. When tyrosine kinase-negative Met mRNA was micro-injected into two-cell to eight-cell stages Xenopus embryos, the liver development was mostly impaired and structures of pronephros and the gut were grossly underdeveloped in the restricted, late stage of development. These results strongly suggest that functional coupling between HGF and Met is essential for the development of internal organs originated from primitive gut and possibly involved in embryonic skeletogenesis. Together with developmental abnormality in mice mutated with HGF or Met gene, essential role of HGF for liver development is highly conserved from amphibian to mammalian species. © 1997 Academic Press

INTRODUCTION

Receptor protein kinases and their ligands plays a crucial role in the coordination of cell to cell interactions involved in the cell growth regulation, differentiation, metabolic responses, cell movement, morphogenic events, etc. During invertebrate and vertebrate development, these molecules regulates pattern formation and inductive tissue interactions. In the amphibian Xenopus embryo, it has been established that several members of fibroblast growth factor and transforming growth factor-β families play key roles in mesoderm induction (1). Likewise, recent studies indicated that noggin, chordin, and follistatin, expressed in the organizer region, are involved in neural inductive processes through regulating activities of bone morphogenic protein and activin (2, 3). Subsequent to neural induction, secondary inductive processes proceed, which successively results in organogenesis and skeletogenesis. Peptide growth factors and their receptors are likely to sequentially govern these successive inductive processes during tissue specification and organogenesis, however, molecular mechanisms on these processes have remained to be addressed.

Met heterodimeric membrane-spanning tyrosine kinase is a specific receptor for hepatocyte growth factor (HGF) (4, 5). HGF, originally identified as a potent mitogen for mature hepatocytes (6), is a four kringle-containing growth factor which exhibits mitogenic, motogenic, and morphogenic activities for a wide variety of cells (7-10). The binding of HGF to Met evokes phosphorylation in C-terminal tyrosine residues, so-called multiple docking site which gathers intracellular signalling molecules, which in turn transduce intracellular signals, leading to mitogenic, motogenic, and morphogenic responses (11). Physiologically, HGF acts as a "trophic factor" for regeneration of the liver, kidney, and lung following tissue injuries and diseases (10). Likewise, HGF has neurotrophic function to survive neurons (12) and also has potent angiogenic activity (9, 10).

Together with unique multipotent characteristics of HGF, the preferential expression patterns of HGF and Met during embryogenesis meant that HGF is likely to be a mesenchymal-derived paracrine mediator for cell-cell interactions between epithelial and mesenchymal tissues during embryonic organogenesis (13-16). The particular importance of HGF and Met in developmental processes was demonstrated by targeted mutation of HGF or Met gene (17-19). HGF is essential for

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the development of the liver and placenta, and HGF supports migration of myogenic precursor cells to limb mesenchyme (19). A role of HGF in subsequent developmental processes were obscure in these knock-out mice due to embryonic lethality caused by impaired placental development, however, in vitro analysis showed that HGF supports morphogenic events during development of the kidney (20, 21), mammary gland and tooth (22-24).

We previously cloned Xenopus cDNAs for both HGF and Met and analyzed expression of these genes during embryogenesis (15, 25). Both the ligand and its receptor, HGF and Met genes are structurally conserved in Xenopus and their expression pattern implicates that HGF-Met system may be involved in early organogenesis during Xenopus development. To specify a role of HGF in Xenopus embryogenesis, we here generated a tyrosine kinase-negative Met mRNA and introduced it into the embryo. Such a dominant expression of tyrosine kinase-negative Met resulted in developmental deficiency in internal organogenesis. Our results indicate that functional coupling between HGF and Met is essential for the development and morphogenesis of internal organs during Xenopus embryogenesis.

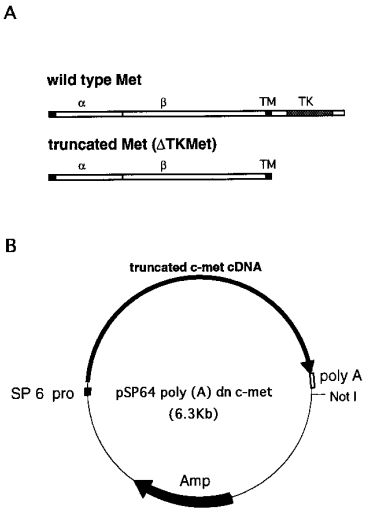

FIG. 1. Micro-injections of truncated Met mRNA in Xenopus embryo. (A) Schematic representation of the structure of the normal (full-length) Met receptor and the truncated Met (ΔTKMet). The transmembrane domain is indicated by a closed box. The stippled region denotes the tyrosine kinase domain. (B) Schematic structure of pSP64 poly A vector for in-vitro synthesis of ΔTKMet mRNA. TM, transmembrane domain; TK, tyrosine kinase domain; SP6 pro, SP6 promotor.

MATERIALS AND METHODS

Preparation of Xenopus embryos. Xenopus laevis adults were purchased from Hamamatsu Seibutsu Kyouzai Co. Ltd. (Hamamatsu, Japan). These embryos were prepared by following two general methods, the artificial and natural fertilization methods. The jelly coat of the embryo was removed by treatment of 3% cysteine-hydrochloride/MMR (pH 7.3-7.4). The stages of Xenopus embryonic development were determined according to the normal stage table as described elsewhere (26).

Plasmids construction. All constructs used for in-vitro transcription were inserted into a pSP64polyA (promega) vector and a modified pSP64polyA vector which was constructed by insertion of a NotI adaptor into an EcoRI site. cDNA for tyrosine kinase-negative Met (ΔTKMet) was constructed by deleting the intracellular region (Fig. 1A). The EcoRI fragment of Xenopus c-met cDNA, encoding the entire extracellular domain, was blunt-ended and inserted into Sma I site of pSP64poly A vector (Fig. 1B). Wild type c-met vector was constructed with the modified pSP64polyA vector. mRNAs for ΔTKMet and wild type Met were synthesized in vitro by SP6 RNA polymerase, using these vectors as templates.

Micro-injection of mRNA into Xenopus embryos. Micro-injection experiments in this report were carried out according to the protocol described elsewhere (27). We usually used a micro-manipulator (Narishige Co.) and microinjector 5242 (Eppendorff). In-vitro synthesized RNA was purified on a sephadex G-50 column. Concentration of the injected RNA was 50-400 ng/ml and the volume for micro-injections were 1-20 nl per embryo.

Histology and staining of embryo. Xenopus embryos reaching the stage of internal organogenesis and the stage of tadpole were fixed in Bouin's solution, dehydrated and blocked in paraffin. The embryos were sectioned into 6 μm and stained with haematoxylin- and eosin-solution. The active staining of β-galactosidase were carried out as described elsewhere (27).

RESULTS

Micro-injection of truncated Met mRNA. To elucidate the role of HGF and Met in Xenopus embryogenesis, we attempted to introduce into the Xenopus embryo a Xenopus truncated Met (ΔTKMet), lacking the cytoplasmic region which covers the tyrosine kinase domain. We reconstructed a truncated Met gene (ΔTKMet gene), which was then transcribed to generate synthetic mRNA (Fig.1A). Micro-injections of synthetic mRNA were carried out at the two-cell to eight-cell stage and the synthetic mRNA was separately injected either into all the animal blastomeres or into all the vegetal blastomeres. We first tested our experimental systems by micro-injection of synthetic mRNAs encoding β-galactosidase followed by whole-mount active staining of the embryos. Almost all embryos so injected developed normally (Fig. 2A, B). In the case of animal injections, the head, skin, and neural tissues of the embryos were specifically stained (not shown). Conversely, in case of the vegetal injections of β-galactosidase mRNA, the ventral and posterior regions were actively stained (not shown). Thus, the relationship between the injected blastomeres and the stained tissues was consistent with the result of cell-fate maps already reported (28). We next micro-injected the synthetic ΔTKMet mRNA in the same manner as in the β-galactosidase mRNA. Although embryos injected with 800 pg the ΔTKMet mRNA into the animal blastomeres developed normally with no abnormality of the external appearance of these embryos (Fig. 2C), micro-injections

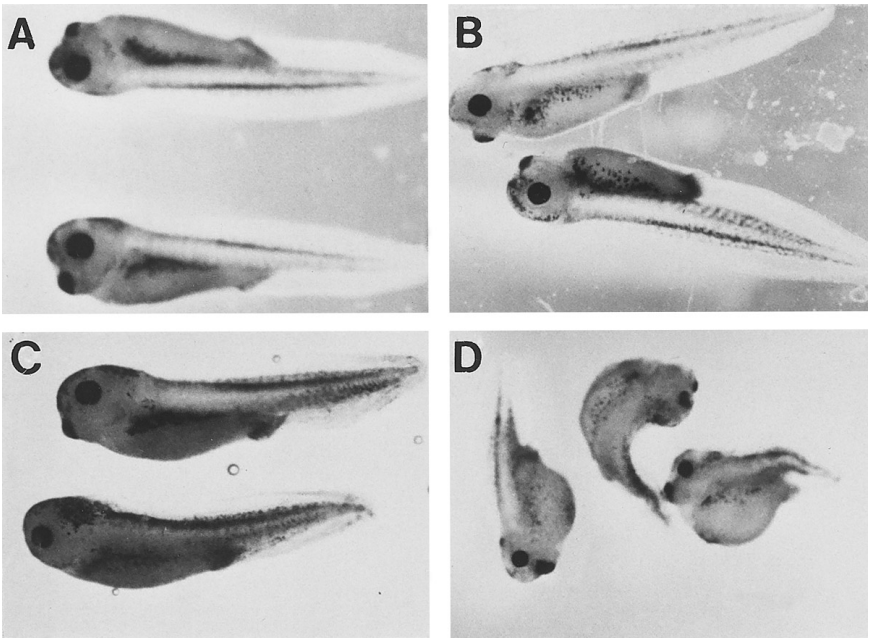

FIG. 2. Effects of the truncated Met mRNA on Xenopus embryo. The injected embryos were allowed to develop until early tadpole stage (A, B, C and D). Control embryos injected with 800 pg of β-galactosidase mRNA into animal (A) or vegetal (B) blastomeres at 4 cell stage, had a normal phenotype. Embryos injected with 800 pg of ΔTKMet mRNA into animal blastomeres at the 4-cell stage also had a normal phenotype (C). Embryos injected with 800 pg of ΔTKMet mRNA into vegetal blastomeres at the 4-cell stage had defects in ventral and posterior regions (D).

of the same amount of ΔTKMet mRNA into the vegetal blastomeres of Xenopus embryo resulted in marked defections of ventral and posterior structures of the embryos, at substantial frequencies: eighty-three percent of embryos injected with 800 pg of ΔTKMet mRNA had defective phenotypes (n=23). In these embryos injected with ΔTKMet mRNA from the vegetal side, growth of the internal organs and elongation of the tail were grossly immature (Fig. 2D), and some embryos had no tail structure whatever (data not shown). In control experiments, there was no apparent abnormality in embryos injected with the same amount of 800 pg β-galactosidase mRNA (a few embryos had an abnormal external appearance) (Fig. 2A, B).

Dose-dependent effect of the truncated Met mRNA. Next, we directed special attention to the specific phenotype produced by vegetal side injection of ΔTKMet mRNA. When we increased the dose of the injected mRNA, the occurrence of a typical abnormal phenotype increased in the dose dependent manner (Fig. 3A to D, F). While the abnormality was more extensive when a higher dose was given, the abnormal phenotype was persistent (Fig. 3A to D, F). Although the injection of ΔTKMet mRNA led to the severe defects in almost all embryos (with the frequency of over 60 % at 600 pg), control injections of 800 pg of β-galactosidase mRNA mostly led to no defects (Fig. 3E, F). Therefore, defects in embryos injected with ΔTKMet mRNA were not related to the nonspecific effects of RNA injections. These results are taken to mean that the molecule introduced exogenously acts with high level specificity in the Xenopus embryo. The phenotype produced by the ΔTKMet mRNA was attributed to the specific effects of the exogenously introduced molecule, truncated Met protein.

Phenotypes of defective embryos injected with the truncated Met mRNA. When vegetal blastomeres of embryos were injected with ΔTKMet mRNA, these tadpoles had marked defects in the internal organs, especially the liver, pronephros and gut (Fig. 4A, C, E and G). In comparison to the specific abnormalities observed in the internal organs, these embryos had a normal head, eyes, heart, neural tubule, notochord (Fig. 4A, C) and circular systems, hematopoietic cells (data not shown) as seen in the normal embryo (Fig. 4B, D). The ventral internal structure of the defected embryos was abnormal, and structure of the gut was markedly immature (Fig. 4A, C, E and G). Detailed analysis of histological cross sections showed no formation of a liver (Fig. 4C and E), and there was little or no formation of the pronephros tubules and a gut tubule (Fig. 4C, G). The epithelial cell layer of the gut was very thin and the number of kidney tubules was decreased as compared with findings in the normal embryos (Fig. 4C, G).

DISCUSSION

Dominant-negative strategy for transmembrane receptors truncated with their intracellular domains has

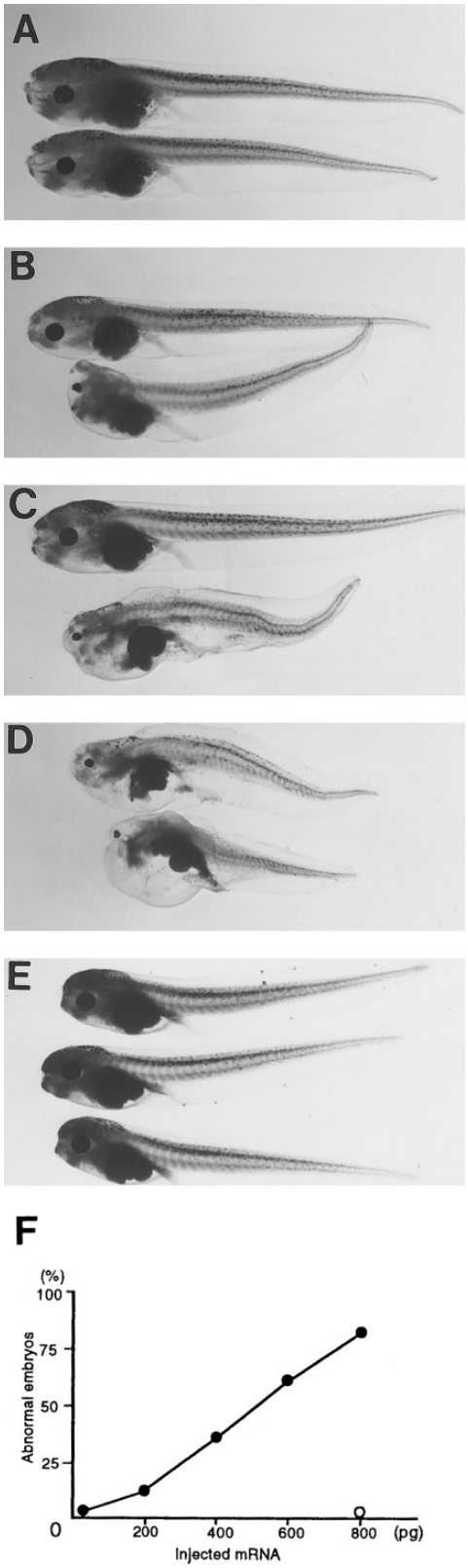

FIG. 3. Dose-dependent increase of abnormal embryo by ΔTKMet mRNA expression. Increasing doses of ΔTKMet mRNA were injected into vegetal blastomeres in 4-cell stage embryos. Occurrences of typical embryos, (A) 20 pg of ΔTKMet mRNA; (B) 200 pg of ΔTKMet mRNA; (C) 400 pg of ΔTKMet mRNA; (D) 800 pg of ΔTKMet mRNA; (E) 800 pg of β-galactosidase mRNA. (F) Frequency of abnormal phenotypes indicated in A-E. Embryos were micro-injected with ΔTKMet mRNA (closed circles) in doses ranging from 20 pg to 800 pg or β-galactosidase mRNA (open circle) in a dose of 800 pg, but the amount of the injected RNA solution were constant for each dose. Over twenty embryos were used for each experimental condition.

successfully been used in Xenopus embryos (29). To determine the specific functions of HGF and its receptor, Met tyrosine kinase in Xenopus embryogenesis, we injected truncated tyrosine kinase-negative Met (ΔTKMet) mRNA in Xenopus embryos. There was no significant effect on the process of embryogenesis when ΔTKMet mRNA was injected into animal blastmeres, however, injection of ΔTKMet mRNA into vegetal blastmeres specifically blocked internal organogenesis and tail elongation, and these morphological changes were not observed until the late tailbud stage in the defective embryo. We previously showed that zygotic expressions of the HGF gene and the Met gene occur from the early neurula stage and the late gastrula stage, respectively, and that these expressions are much higher in ventral than dorsal regions (15, 25). Likewise, Met mRNA is expressed in ventral forgut, posterior tail mesenchyme, and head regions (25). Thus, the regional and temporal occurrence of defected phenotypes due to ΔTKMet coincides with the site of expression of the endogenous Met and HGF gene. The result indicates that the target molecules of ΔTKMet may be the endogenous Met and/or HGF, and thus it is highly probable that ΔTKMet acts as a specific dominant-negative molecule against an endogenous Met in Xenopus embryo. Therefore, Xenopus embryos expressing ΔTKMet seem to be counterpart of loss-of-function mutant mice introduced by targeted mutation of HGF or Met gene (17-19). Most strikingly, grossly defected liver development in Xenopus embryo expressing ΔTKMet was highly compatible with $HGF^{-/-}$ or $Met^{-/-}$ mutant mice. Essential role of HGF, originally identified as a potent mitogen for mature hepatocytes, is therefore highly conserved from amphibian to mammalian species.

Epithelial-mesenchymal interactions mediate crucial aspects of normal development, affecting tissue induction, organogenesis, and morphogenesis of specific multicellular structures. Requirements of particular embryonic mesenchymes for the processes of the vertebrate epithelial histogenesis have been demonstrated for the kidney, pancreas, lung, liver, mammary gland, cartilage, bone and teeth, over various species (30). Epithelial cells of the developing liver, lung, pancreas, kidney, and tooth, express Met, while the surrounding mesenchymal cells express HGF gene (13, 20, 24 and our unpublished results). In vitro experiments using tissue rudiments and embryonal cells also suggested

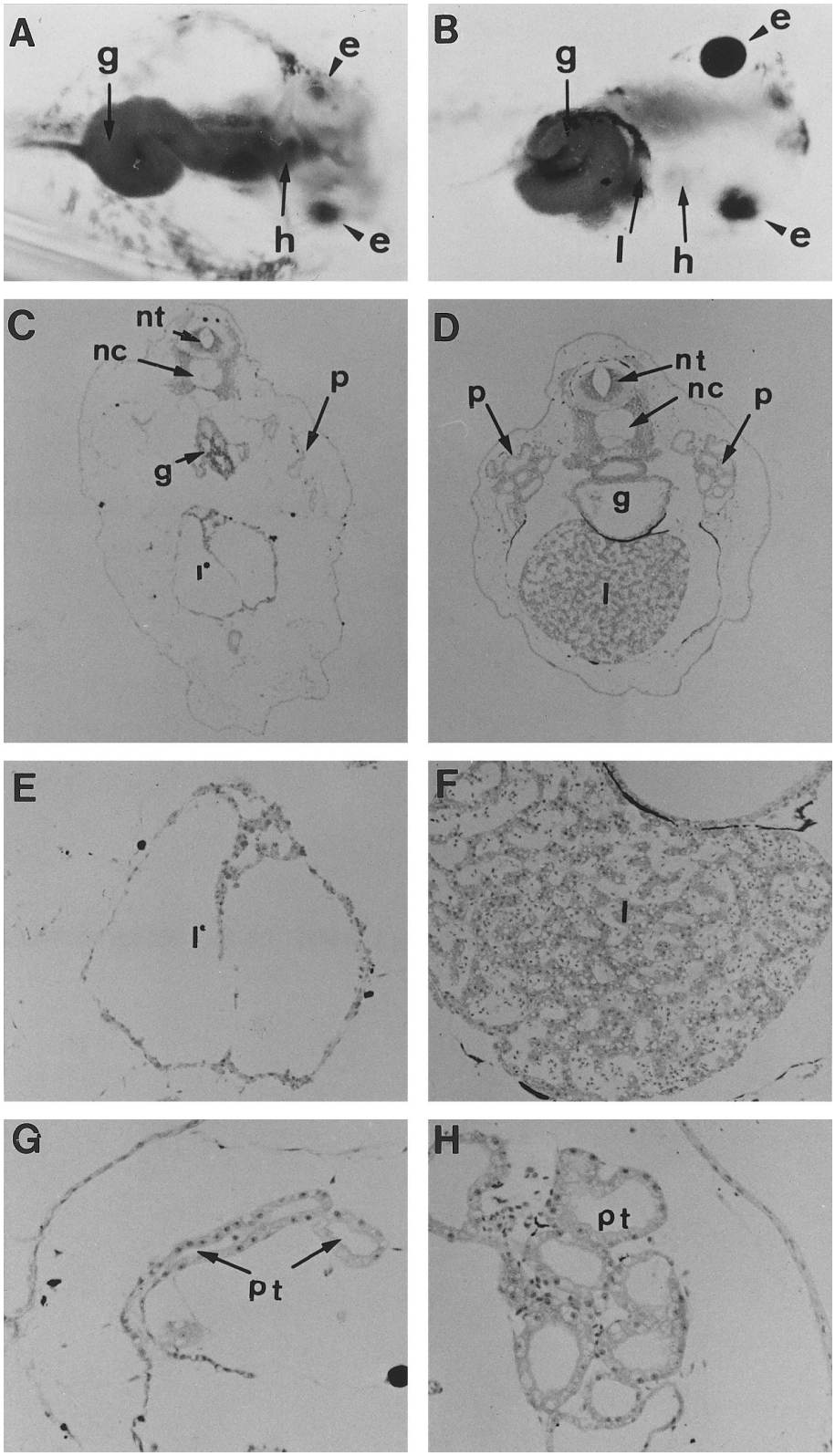

FIG. 4. External appearance and histological cross section of defected embryo injected from the vegetal side with ΔTKMet mRNA. Ventral views of an typical embryo injected with 800 pg of ΔTKMet mRNA into vegetal blastomeres at the 4-cell stage and a normal embryo is indicated in (A) and (B), respectively. Transverse sections of a stage 44 embryo injected with ΔTKMet mRNA (C, E and G) and a normal embryo (D, F and H) are shown. Close-up views of liver of the ΔTKMet introduced embryo (E) and the liver of a normal embryo (F), and close-up views of the developing kidney of the ΔTKMet introduced embryo (G) and a normal embryo (H) are respectively shown. Transverse sections were prepared from the midtrunk region. e; eye, g; gut, h; heart, l; liver, l*; liver synonym, nc; notochord, nt; neural tube, p; pronephros, pt pronephros tubule.

the involvement of HGF in the morphogenic epithelial-mesenchymal interactions during organogenesis of the kidney (20, 21), tooth (24), and lung (our unpublished result). HGF induces a branching-duct like structure and lumen formation for epithelial cell lines derived from kidney, liver and mammary gland (20-23, 31-33). Together with multipotent features of HGF, these results suggest that HGF-Met system is responsible for signal exchange between epithelial and mesenchymal (or stromal) tissues in mammalian systems. Our present result indicates that roles of HGF as a mediator in specific cell-cell interactions for organogenesis seem to be conserved also in amphibian Xenopus. In Xenopus embryo, HGF is expressed in the mesoderm surrounding the endodermal epithelium (15), while Met mRNA is expressed in ventral forgut, posterior tail mesenchyme, and head regions (25). Our functional study using dominant-negative strategy clearly indicates that HGF-Met system play a crucial role for organogenesis and morphogenesis of the liver, pronephros, and gut, mediating specific cell-cell interactions during Xenopus embryogenesis. It is noteworthy, however, that apparent abnormality in the development of pronephros and gut in Xenopus embryo with loss-of-fucntion mutation of Met is distinct from the knock-out mouse of HGF or Met gene. In the knock-out mouse, the morphological appearance of tubular epithelia and lumen structures in the internal organs is normal (17-19). Except for the liver, specific ligand-receptor systems distinct from HGF-Met system may possibly play redundant function for development of tubular and ductular structures during mammalian organogenesis.

Defected liver development in embryos subjected to loss-of-function mutation in Xenopus and mice raises how HGF is involved in liver development. The progenitor cells of the liver parenchyma develops from ventral endodermal tissue through an inductive signal from neighbouring cardiac mesoderm (34). One possible explanation may be that HGF itself functions as mesoderm-derived inductive as well as instructive signal for the specification of endodermal cells toward liver progenitor cells. The other seems to be that HGF functions as general mesoderm-derived factor which triggers subsequent cell growth and morphogenic events in parenchymal cells derived from endodermal tissue, after the initial inductive process toward hepatic specification. Impaired development of both the liver and gut in affected Xenopus embryo implicate that HGF may preferably triggers cell growth and morphogenic events, following instructive specification of endodermal tissue, however, this issue remains to be addressed.

In addition to developing internal organs, both HGF and c-met gene are expressed in developing limb bud, branchial arches, ribs and somites during mammalian and avian embryogenesis (13, 14, 35, 36). These expression patterns suggested the possible involvement of HGF and Met in embryonic skeletogenesis, a notion supported by previous in vitro experiments. Articular chondrocytes express the Met and HGF stimulates motility, proliferation and proteoglycan synthesis of chondrocytes (35). Our present finding that the truncated Met specifically blocks outgrowth of the tail in the embryo also suggests the possible involvement of HGF in embryonic skeletogenesis in the Xenopus embryo. HGF is also known to partly act as a neural inducer in early chick embryogenesis (16, 37), and HGF functions as a novel member of neurotrophic factor and also as axonal chemoattractant for developing motor neurons (12, 38). However, we detected no defects in neural tissues of embryos injected with ΔTKMet mRNA. The lack of abnormality in neural tissue formation seems to be consistent with the results of targeted disruption of HGF or Met in the mouse embryo.

In conclusion, functional coupling between HGF and Met confer a critical intercellular signal, which governs the development and morphogenesis of the liver over a wide range of species. Although in vitro experiments have demonstrated unique morphogenic functions of HGF during epithelial morphogenesis in various cells and tissue, we have shown for the first time that HGF is an essential molecule for epithelial morphogenesis during organogenesis in vivo. HGF and Met are highly conserved molecules not only structurally but also functionally, in mediating cell-cell interactions for the construction of normal tissue structures during organogenesis, as well as tissue regeneration.

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