# Bovine hepatocyte growth factor and its receptor c-Met: cDNA cloning and expression analysis in the mammary gland

Running title: Cloning of bovine HGF and c-Met

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**Abstract**

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic cytokine that plays a crucial role in the embryonic and postnatal development of various organs including the mammary gland. We cloned bovine HGF and its c-Met receptor cDNAs, and examined their expression during mammary gland development in dairy cows. The 2.5-kbp HGF cDNA clone contained a 2,190 bp open reading frame coding a 730 amino acid protein, while the 4.8-kbp c-Met cDNA clone contained a 4,152 bp open reading frame coding a 1,384 amino acid protein. The bovine HGF and c-Met sequences exhibited more than 87% identity with those of other mammals. RT-PCR analysis revealed ubiquitous expression of both HGF and c-Met mRNAs in various bovine tissues tested. HGF mRNA was detected only in the inactive stage of bovine mammary gland development and not in the developing, lactating, and involuting stages, while c-Met mRNA was detected in the inactive and involuting stages. Immunohistochemical analysis demonstrated that the c-Met protein was found on mammary epithelial cells in the inactive, developing, and involuting stages, and on myoepithelial cells in all stages. These results suggest pivotal roles of HGF and c-Met in the development of bovine mammary gland.

**Keywords:** HGF, c-Met, cDNA cloning, mammary gland, epithelial cells

## Introduction

The mammary gland is one of the unique organs in which cycles of morphogenic and functional change accompany the process of pregnancy, lactation and involution in postnatal life [1]. The mammary gland in the inactive state consists of slightly branched ducts embedded in connective stromal tissue. Following the onset of pregnancy, the development of the mammary gland begins with ductal branching and alveolar morphogenesis of epithelial cells. Subsequently, alveolar epithelial cells differentiate functionally to produce and secrete milk, and to be maximally activated during lactation. After weaning or upon cessation of milking, the mammary gland involutes, leaving only the rudimentary ductal system and several alveoli. The events associated with mammary gland development are highly regulated by circulating hormones [1,2], and it has been established that several growth factors are produced in the mammary gland under the control of systemic hormones and that these are involved in mammary gland development in rodents [2].

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a multifunctional cytokine derived from stroma that induces cell proliferation, differentiation, and motility in a variety of epithelial cells by binding to the product of the *c-met* protooncogene [3,4]. HGF and c-Met have also been reported to be involved in the embryonic and postnatal development of a variety of tissues, including that of the mammary gland [3, 4]. While HGF induced tubulogenesis has been reported in mouse mammary epithelial cells *in vitro* and *in vivo* [5-9], limited information about the function of *hgf* and *c-met* genes in ruminants is currently available [10-12].

In the present study, we determined the full-length sequences of the coding region for bovine HGF and c-Met mRNAs, and analyzed their expression patterns during the process of mammary gland development in cows.

## Materials and Methods

### Animals

Experimental procedures were in accordance with the guidelines as set out by the Animal Care and Use Committee at Hokkaido University and the Laboratory Animal Control Guidelines of the National Institute of Animal Health. Fifteen non-pregnant Holstein cows that had not been milked for more than a year were used to induce development of the mammary gland and consequent lactation as previously reported [13, 14]. Six cows were sacrificed without any treatments and several tissues including the mammary gland in inactive stage were obtained. Nine cows were received repeated injections of estradiol-17β and progesterone to develop the mammary gland toward the onset of lactation, and three of these were sacrificed to obtain the mammary glands in developing stage. The remainders of the cows were received additional injections of reserpine to achieve full lactation, and three of these were sacrificed to obtain the mammary glands in lactating stage. The rest of the cows were sacrificed after they minimized milk production to obtain the mammary glands in involuting stage. Tissues were frozen in liquid nitrogen and stored at -80°C until use.

### Cloning and expression analysis of bovine HGF and c-Met cDNAs

Bovine HGF and c-Met cDNAs were cloned from total RNA extracted from bovine mammary cells [15] by reverse transcription-polymerase chain reaction (RT-PCR) and 3' rapid amplification of cDNA ends (3' RACE) methods [16] (supplement Fig. S1).

To examine the expression of bovine HGF and c-Met, total RNA (2 μg) extracted from various tissues was reverse transcribed and subjected to PCR. The primers used for bovine HGF corresponding to the cloned sequences (+1,936 to +2,286) were as follows: Forward: 5’-TACCTAATTATGGGTGCACAATTC-3’, Reverse: 5’-TCCATTTTGCATAATATGCCACTC-3’. The PCR profile used was 35 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Primers used for bovine c-Met corresponding to the cloned sequences (+3,140 to

+3,400) were as follows: Forward: 5'-CCCAACTACAGAATGGTTTCCC-3', Reverse: 5'-ATCAGACTGCTCGGCCCAATTA-3'. The PCR profile of 35 cycles was used with denaturation at 94°C for 30 sec, annealing at 58°C for 1 min, and extension at 72°C for 1 min. PCR products were subjected to electrophoresis in a 2% agarose gel containing 0.01% ethidium bromide. As references, glyceraldehyde-3-phosphate dehydrogenase (G3PDH; GenBank Accession Number U85042; +490 to +942; Forward: 5'-ACCACAGTCCATGCCATCAC-3' and Reverse: 5'-TCCACCACCCTGTTGCTGTA-3') and β-casein (GenBank Accession Number NM181008; +120 to +672; Forward: 5'-CTCAATGTACCTGGTGAGAT-3' and Reverse: 5'-AGGCCTGAATGGGCATATCTCT-3') mRNAs were also analyzed.

**Immunohistochemistry**

Cryostat sections of 4 μm in thickness were prepared and mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Micro Slides, Muto Pure Chemicals, Tokyo, Japan). The signals were generated using a Histofine kit (Nichirei, Tokyo, Japan) according to the manufacture's instructions. Briefly, the sections were fixed in cold acetone for 10 min and permeabilized in 0.2% Triton X-100 in PBS for 30 min. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 30 min. Nonspecific antibody binding was blocked with 3% normal rabbit serum for 1 hr. The sections were incubated with a primary antibody against either human c-Met (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; diluted 1:200) or human HGF (American Research Products, Inc., Belmont, MA, USA; diluted 1:50-200) overnight. The sections were then treated with a biotinylated goat anti-rabbit antibody followed by the application of avidin-horseradish peroxidase complex. The antigen-antibody reaction was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.002% $H_2O_2$. The sections were counterstained with hematoxylin.

## Results

The entire sequences of the coding regions for bovine HGF and c-Met mRNAs were determined (GenBank Accession Numbers AB110822 and AB112434, respectively). The cloned bovine HGF cDNA consisted of 2,492 nucleotides and contained a open reading frame of 2,190 bp encoding 730 amino acids. The cloned bovine c-Met cDNA consisted of 4,823 nucleotides with a open reading frame of 4,152 bp encoding 1,384 amino acids (supplement Fig. S2). The deduced amino acid sequences of these genes showed more than 87% identity with the same proteins in humans and rodents, demonstrating the marked conservation of the characteristic motifs of these genes (supplement Table S1 and Fig. S2).

mRNA expression of HGF and c-Met was then examined by RT-PCR in various tissues from non-pregnant Holstein cows. HGF and c-Met mRNAs were found in many organs including the liver, lung, heart, spleen and mammary gland (Fig. 1A). Their mRNAs were not detected in the pancreas, but this might be due to degradation of RNA in this organ as seen in the expression of G3PDH mRNA. The expression of HGF and c-Met mRNA in the mammary gland was investigated further during the course of hormonally-induced development, the status of which was confirmed by the induction of β-casein mRNA (Fig. 1B). HGF mRNA was detected only in the inactive stage and not in the developing, lactating, and involuting stages. c-Met mRNA was detected in the inactive and involuting stages, but not in the developing and lactating stages.

Localization of the c-Met protein in the mammary gland was examined by immunohistochemistry. Interestingly, c-Met immunoreactivity was observed at all stages, albeit at different extents of localization (Fig. 2). More specifically, c-Met protein was observed on epithelial cells in the inactive, developing, and involuting stages, but not in the lactating stage, and at all stages on myoepithelial cells, while it was not found on adipocytes and fibroblasts. The anti-human c-Met antibody used for immunohistochemistry also decteced a single protein with a molecular weight of 140kDa in the membrane fractions of lactating mammary gland and cultured mammary epithelial cells, but not of mammary gland

from a calf (supplement Fig.S3). Conversely, HGF immunoreactivity was not detected using anti-human HGF antibody (data not shown).

## Discussion

In the present study, we elucidated the entire sequence for the coding region of bovine HGF and c-Met mRNA. Here we provide the first evidence of their expression during hormone-induced mammary gland development in dairy cows.

The deduced bovine HGF amino acid sequence and c-Met mRNA sequences indicated that motifs and amino acids that are important for the maintenance of characteristic structural and functional features of the proteins were conserved (supplement Fig. S2). For example, bovine HGF contained four Kringle domains, which are important for HGF binding to c-Met, and bovine c-Met contained a tyrosine kinase domain and a multifunctional docking site essential for c-Met signaling [3, 4]. These results suggest that similarities in the physiological functions of the HGF/c-Met system extend beyond the level of species, which is likely given our previous report in which we demonstrated that c-Met from a bovine cell line could be stimulated by human recombinant HGF [17].

Bovine HGF and c-Met mRNAs were ubiquitously expressed in several tissue types, including the mammary gland (Fig. 1A). The expression pattern of these genes during hormone-induced mammary gland development in cows (Fig. 1B) seems virtually similar to that observed during naturally occurring mammary gland development in rodents [5, 8, 18-20]. As the hormone injections altered the expression pattern, these genes in cow might be regulated directly by ovarian hormones such as estradiol-17β and progesterone as in rodents [9, 21, 22].

The restricted expression of c-Met protein was observed on mammary epithelial cells and myoepithelial cells, but not on stromal adipocytes and fibroblast, in the inactive mammary glands. The former was confirmed by the detection of 140kDa protein in cultured mammary epithelial cells. Moreover, c-Met protein on mammary epithelial cells, but not myoeipthelal cells, disappeared in the lactating stages and reappeared in the involuting stage. Quite the same localization of c-Met protein was observed in the lactating and involuting stages of mammary glands of naturally delivered cows (Yamaji et al., unpublished

observation). It is therefore indicated that the regulation of c-Met protein expression during the artificial induction of mammary gland development and involution are virtually the same as that occurred naturally.

However, apparent contradictions exist between the expression pattern of c-Met mRNA and c-Met protein. More specifically, intense expression of c-Met protein was found in the developing stage whereas little amount of c-Met mRNA was detected. Such a low levels of the c-Met mRNA expression may be attributed to rapid degradation c-Met mRNA after cells expressing c-Met are exposed to cytokine and hormones [21] and/or natural decreases during mammary gland development toward the onset of lactation as in rodents [5, 8]. On one hand, the mechanism(s) of sustained expression of c-Met protein from the inactive stage to the developing stage remains to be elucidated.

It has been demonstrated that HGF promotes ductal morphogenesis in mammary epithelial cells *in vitro* and *in vivo* [5-9] and also that recombinant human HGF stimulates the formation of branching tubules in isolated bovine mammary epithelial cells (Yamaji et al., unpublished observation). Considering the restricted expression of c-Met as well as the finding that HGF is mainly produced in stromal cells [3, 4], we propose that HGF may be derived from mammary stromal cells where it then acts on adjacent epithelial cells in a paracrine manner to induce ductal morphogenesis in cows.

In addition, we revealed that the expression of c-Met on bovine mammary myoepithelial cells was constitutive. It was reported that HGF stimulated the proliferation of human mammary myoepithelial cells in an extracellular matrix-dependent manner [23]. Consequently, the HGF/c-Met system might also play an important role in the survival of myoepithelial cells to maintain ductal structure for milk ejection.

In conclusion, we successfully cloned bovine HGF and its c-Met receptor, as well as their expression patterns during the cycle of mammary gland development and involution. These results therefore suggest the role of the HGF/c-Met system in the structural and functional regulation of the mammary gland in cows.

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**Figure captions**

**Figure 1. Expression of HGF and c-Met mRNAs in the bovine tissues.**

(A) Total RNA (2 μg) extracted from various tissues form a non-pregnant cow were subjected to RT-PCR analysis. Shown is a representative result of three cows. (B) Total RNA extracted from the mammary glands at different stages of development under hormonally induced lactation of cows were subjected to RT-PCR analysis. Shown are representative results of each stage of mammary gland development.

**Figure 2. Immunohistochemical localization of c-Met in the bovine mammary gland.**

Cryostat sections (4 μm) of the mammary tissue samples used in Fig. 1B were stained with hematoxylin-eosin (HE, upper panels), anti-c-Met antibody (middle panels) or normal rabbit serum (lower panels). Results are representative of each stage of mammary gland development. Scale bar: 100 μm.

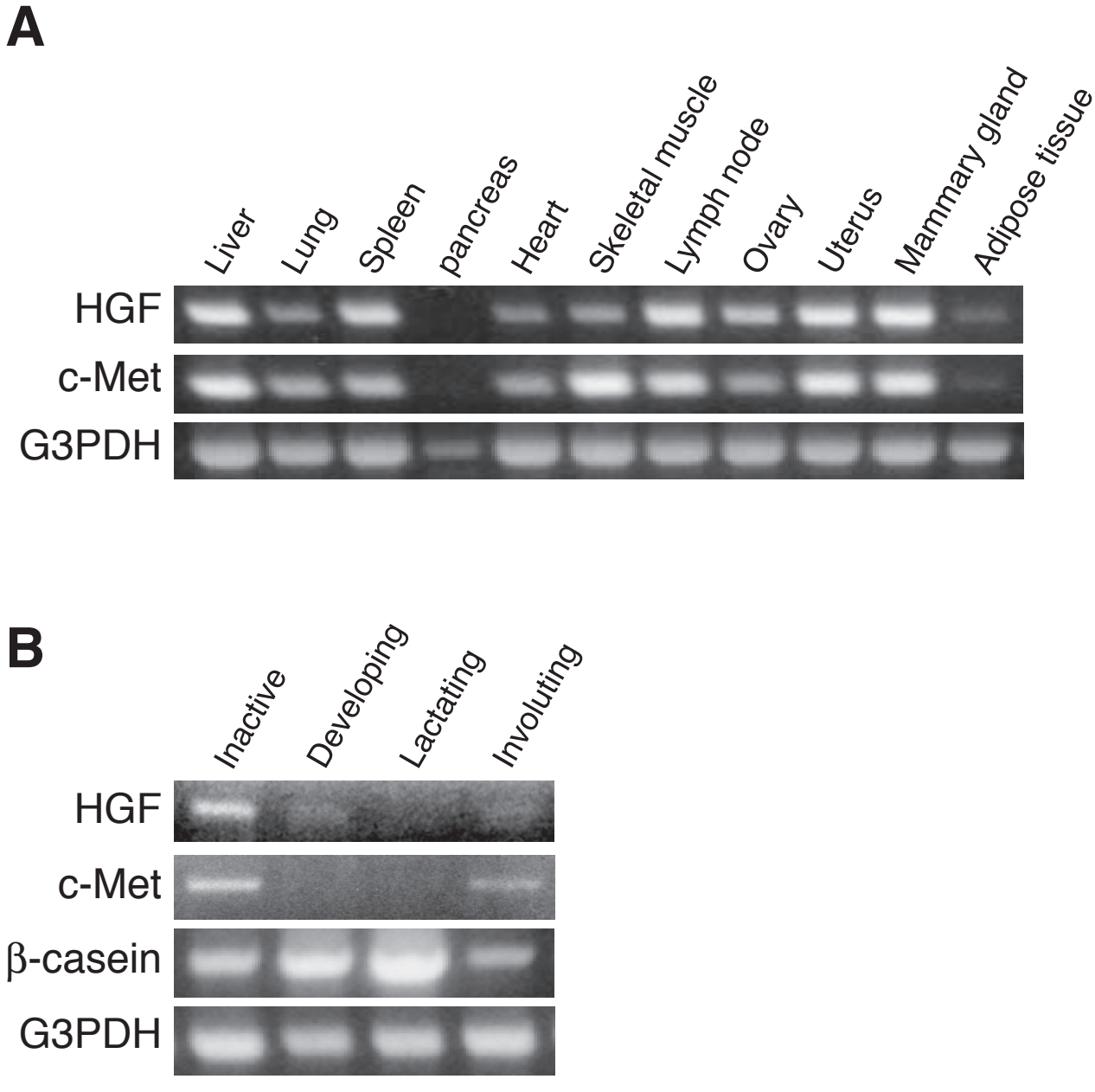



Figure 1 Yamaji et al.

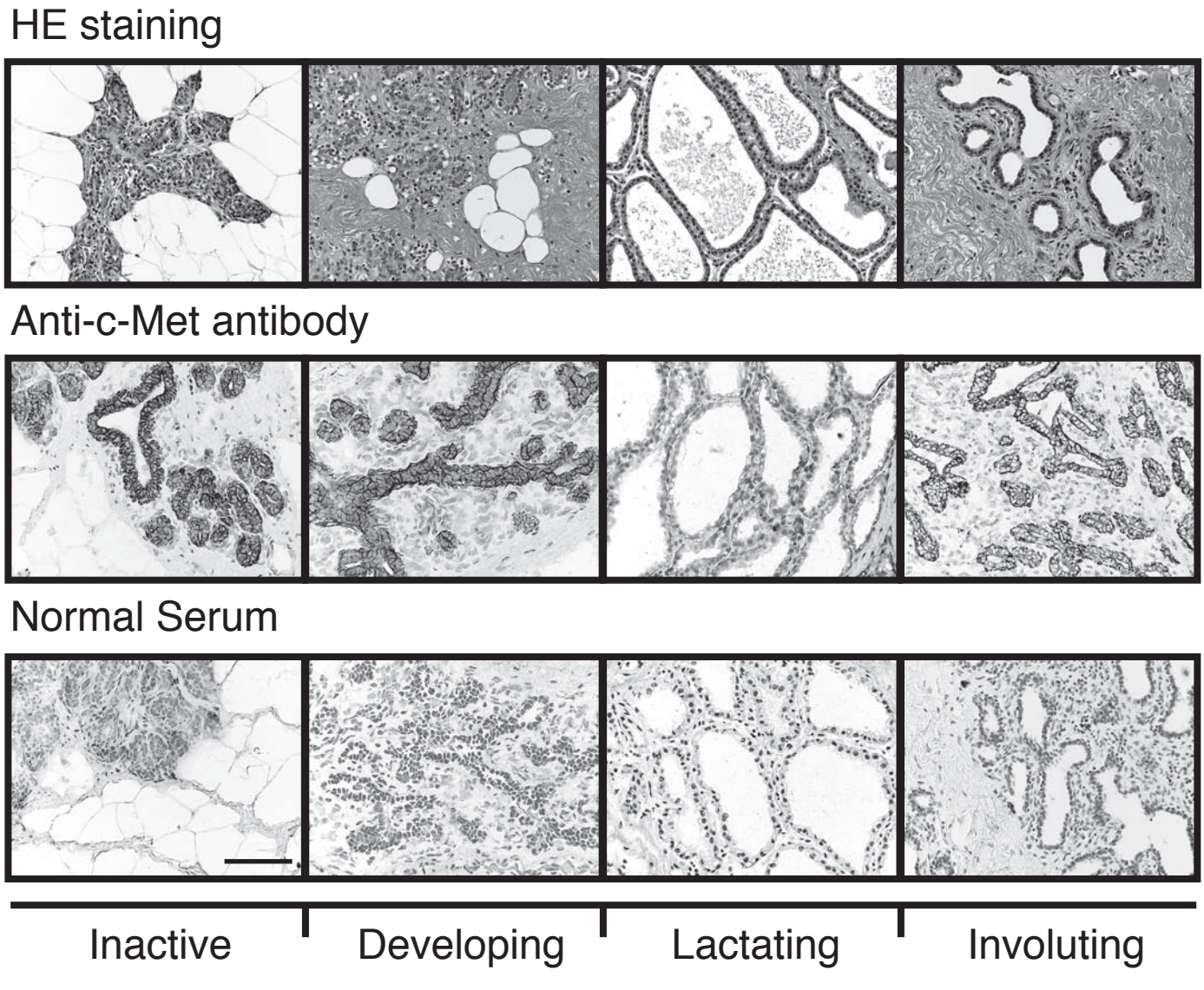



Figure 2 Yamaji et al.

**Supplements**

**Supplementary Table S1.**

**Homology (%) of deduced amino acid sequence of cloned bovine HGF and c-Met to those of other species.**

| | Bovine | Human | Murine | Rat | Feline | Canine |
|---|---|---|---|---|---|---|
| HGF | 100 | 92.5 | 93.3 | 92.7 | 97.4 | 96.6 |
| c-Met | 100 | 90.2 | 88.8 | 87.7 | - | - |

**Supplementary figure captions**

**Figure S1. Cloning strategy of bovine HGF and c-Met cDNAs.**

The coding and untranslated regions of HGF (A) and c-Met (B) cDNAs are shown as an open and closed box, respectively. F1-F4 indicate the cloned fragments of respective cDNAs. The adaptor primer was used for the 3' rapid amplification of cDNA ends (3' RACE) method.

Bovine mammary cells were prepared by enzyme digestion and density-gradient centrifugation with Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) [15]. Bovine HGF and c-Met cDNAs were cloned from total RNA of the bovine mammary cells by reverse transcription-polymerase chain reaction (RT-PCR) and 3'RACE methods as previously described [16]. Total RNA (2 μg) obtained from the mammary cells was reverse-transcribed with oligo-dT primer and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The nucleotide sequences of short cDNA F1 fragments for bovine HGF and c-Met have already been submitted to GenBank (Accession Numbers AB056447 and S72476 for HGF, and AB057406 for c-Met) [10-12]. After the nucleotide sequence for each F1 had been confirmed, the cDNA fragments from an unknown region (F2 to F4) were amplified by PCR with primer sets designed based on sequences reported for human and murine HGF cDNAs (GenBank Accession Numbers XM_168542 and D10213), and human and murine c-Met cDNAs (NM_000245 and NM_008591).

The PCR products were ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA), cloned in Library Efficiency DH5a Competent Cells (Invitrogen) and sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

**Figure S2. Amino acid sequences of bovine HGF and c-Met.**

Deduced amino acid sequences of bovine HGF (A) and c-Met (B) are compared to those reported for other species. Asterisks indicate amino acid residues that are identical

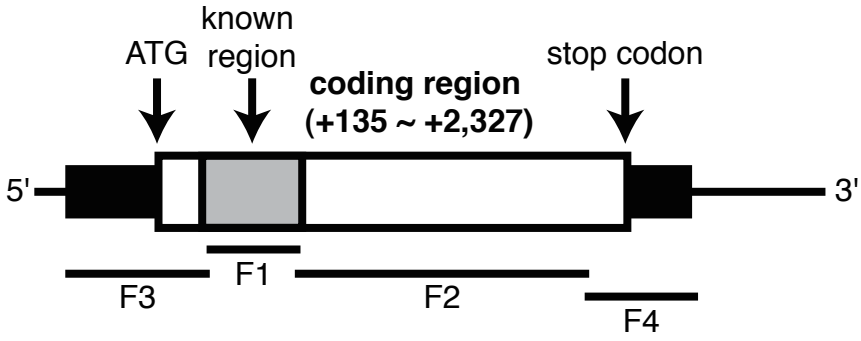
among species. (A) Shaded boxes indicate the four Kringle domains and a trypsin-like serine proteinase domain, respectively. Cysteines enclosed by open boxes are expected to form disulfide bond. (B) Shaded boxes indicate an extracellular semaphorin-like domain (Sema) and an intracellular tyrosine kinase domain, respectively. Open boxes indicate a PSI domain, a transmembrane domain, and a multifunctional docking site, respectively. Gray underlines indicate four typical IPT repeats.

**Figure S3. Western blot analysis of bovine c-Met protein.**

A single protein band with a molecular weight of 140kDa was detected by anti-human c-Met antibody in mammary gland tissue (lane 1) and cultured mammary epithelial cells (lane 3) from cows, but not in mammary gland tissue from calf (lane 2).

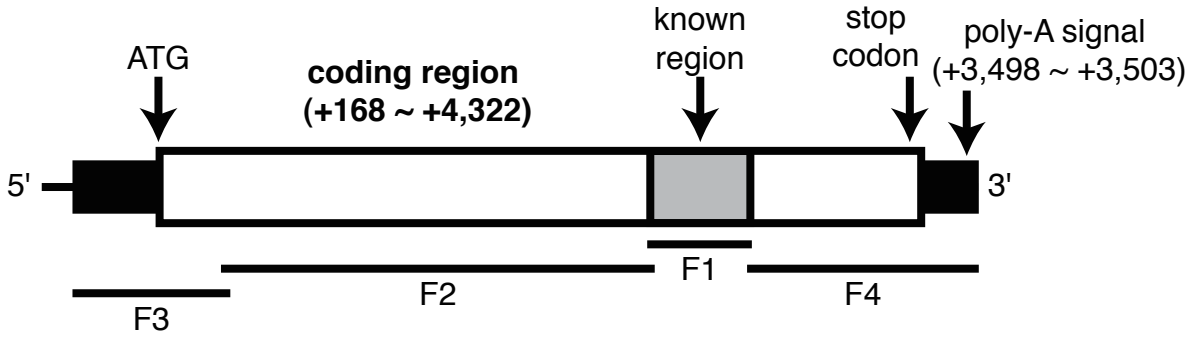
Bovine mammary glands were obtained from a naturally lactating cow and a calf (one month old). Mammary epithelial cells were prepared as described in the caption for Figure S1. The tissues and the cells were separately homogenized in the buffer [20 mM Tris-HCl (pH 7.4), 1 mM EDTA], and the homogenates were centrifuged at 800 x g for 5 min to remove debris and subsequently at 100,000 x g for 60 min to obtain a membrane fraction. The membrane proteins (40 μg) were separated by SDS-PAGE (10% gel) and transferred onto PVDF membrane (Millipore, Bedford, MA, USA). The membrane was immersed in a blocking buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 and 5% skimmed milk] and then anti-human c-Met antibody (Santa Cruz Biotechnology, diluted 1:1,000). The bound antibody was visualized using a horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Zymed Laboratories, South San Francisco, CA, USA, diluted 1:2,000) and an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

A. Cloning of bovine HGF cDNA fragments




| cDNA fragment (nucleotide) | | Primer | Sequence (5'-3') |
|---|---|---|---|
| F1 | (+671 ~ +867) | Forward | ACTGTCGAAATCCTCGAGGGGAA |
| | | Reverse | ATTTGTGCCGGTGTGGTGTCTGAT |
| F2 | (+693 ~ +2,136) | Forward | CCCTGGTGTTTCACAAGCAATCCA |
| | | Reverse | ATCCAATCTTTTCAGCCCCAGCAC |
| F3 | (+1 ~ +796) | Forward | CACACAACAAACTTAGCTCATCGC |
| | | Reverse | TTTCCCCATTGCAGGTCATGCATT |
| F4 | (+1,936 ~ +2,492) | Forward | TACCTAATTATGGGTGCACAATTC |
| | | Reverse | CAAACAAAACAACAGAAAACACCC |

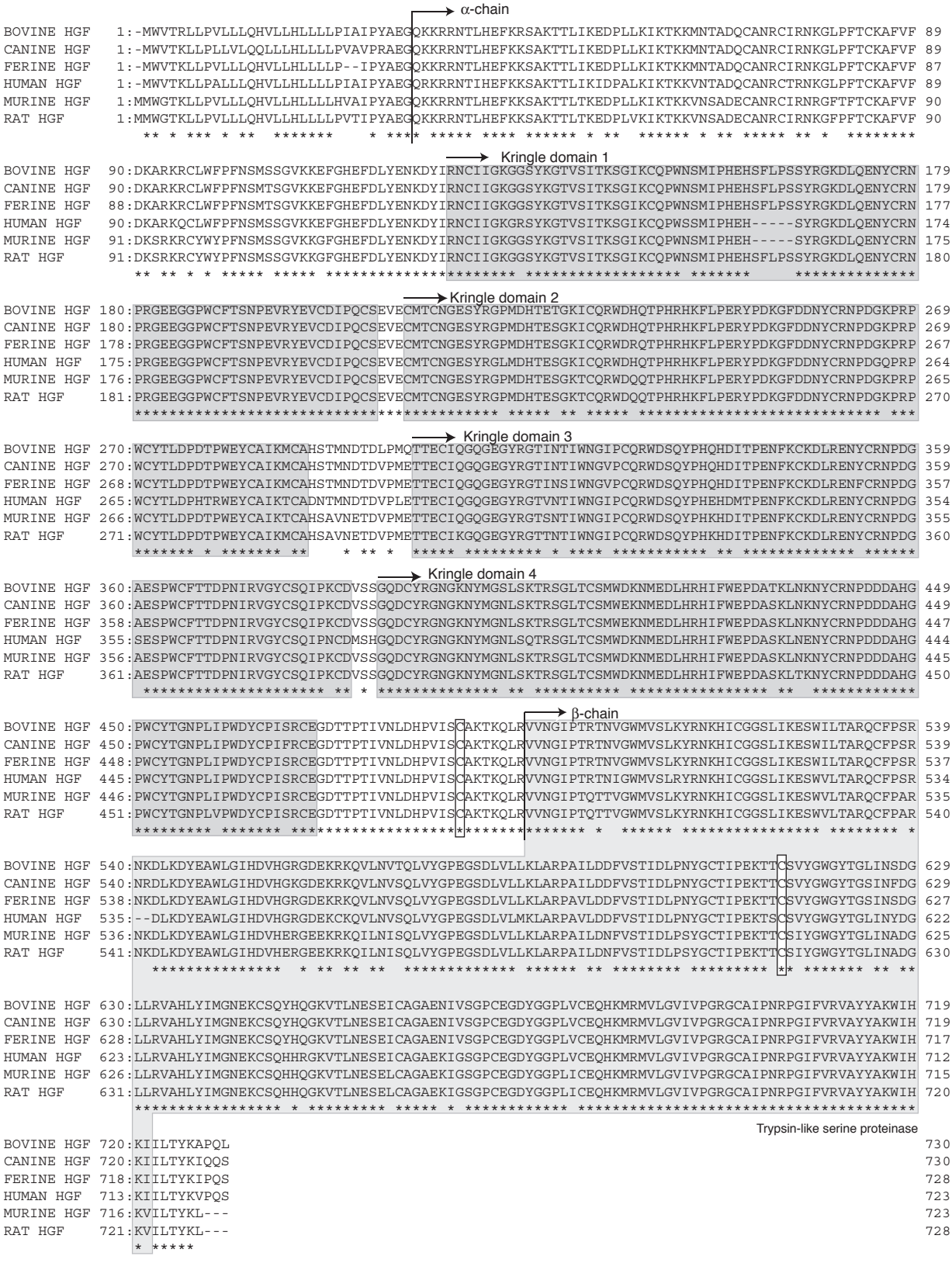
B. Cloning of bovine c-Met cDNA fragments




| cDNA fragment (nucleotide) | | Primer | Sequence (5'-3') |
|---|---|---|---|
| F1 | (+3,140 ~ +3,400) | Forward | CCCAACTACAGAATGGTTTCCC |
| | | Reverse | ATCAGACTGCTCGGCCCAATTA |
| F2 | (+464 ~ +3,361) | Forward | GGACTGCAGCAGAAAGCCAAT |
| | | Reverse | TGGACCAGCTCTGGATTTAGAGC |
| F3 | (+1 ~ +639) | Forward | GAGCGCTTTGTGAGCAGATGC |
| | | Reverse | CCTCCGACTCTATGTCTGCAG |
| F4 | (+3,332 ~ Adaptor) | Forward | CCTCAGTGCTCTAAATCCAGAGCTGGTCC |
| | | Reverse | GGCCACGCGTCGACTAGTAC |

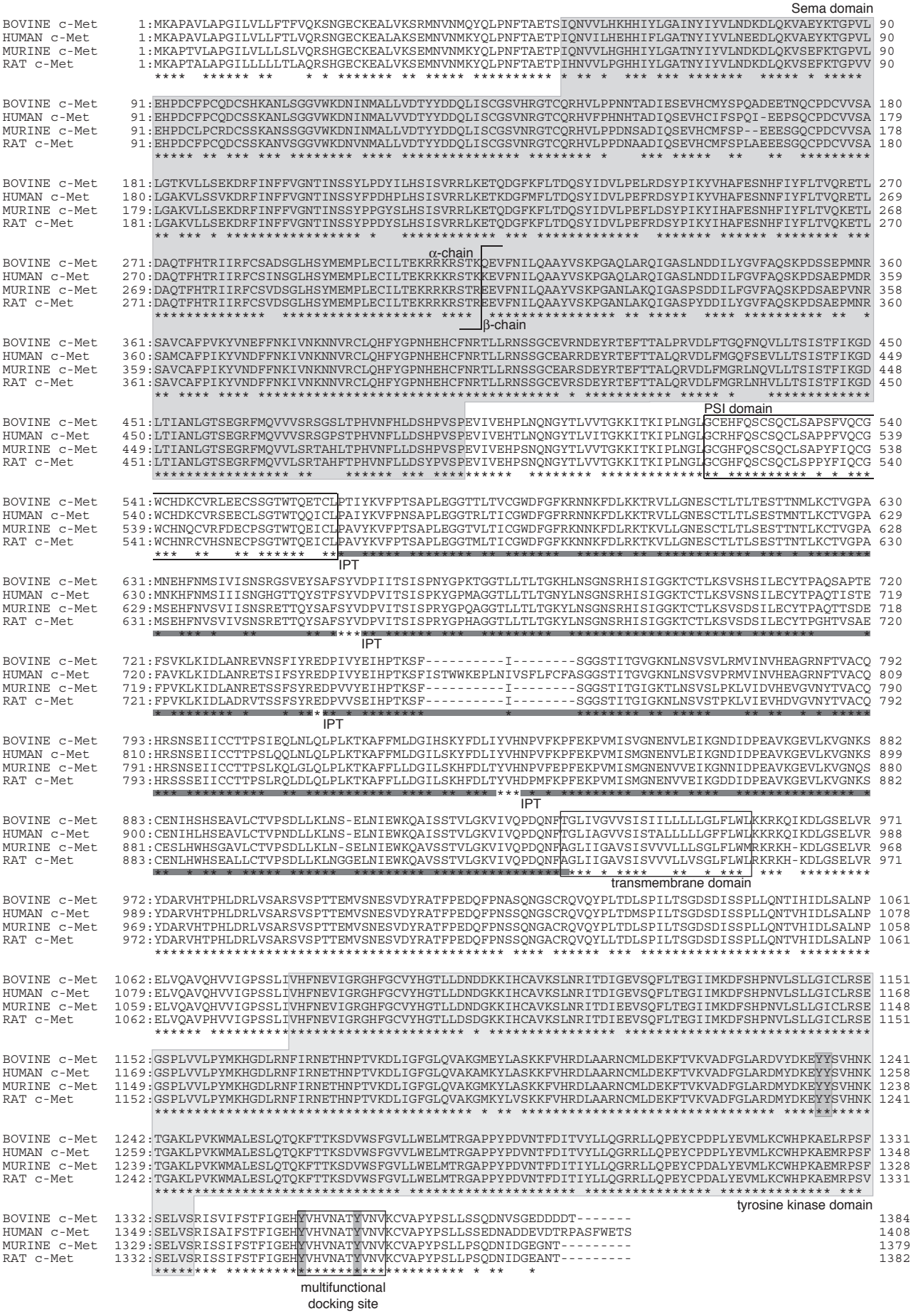
Supplementary figure S1 Yamaji et al.

A. Amino acid sequences of HGF

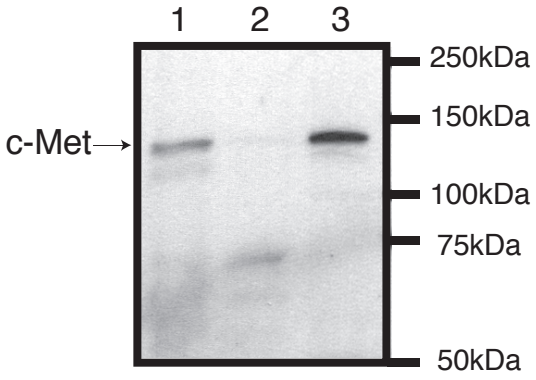



Supplementary figure S2A Yamaji et al.

B. amino acid sequences of c-Met




Supplementary figure S2B Yamaji et al.




Supplementary figure S3  Yamaji et al.