



Short communication

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

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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 2190 bp open reading frame coding a 730 amino acid protein, while the 4.8-kbp c-Met cDNA clone contained a 4152 bp open reading frame coding a 1384 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

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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.

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## 1. Introduction

The mammary gland is one of the unique organs in which cycles of morphologic and functional change accompany the process of pregnancy, lactation and involution in post-natal 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.

## 2. Materials and methods

### 2.1. 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 received repeated injections of estradiol-17 $\beta$  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  $-80^{\circ}\text{C}$  until use.

## 2.2. 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] (Supplementary data, Fig. S1).

To examine the expression of bovine HGF and c-Met, total RNA ( $2\ \mu\text{g}$ ) extracted from various tissues was reverse transcribed and subjected to PCR. The primers used for bovine HGF corresponding to the cloned sequences (+1936 to +2286) were as follows—forward: 5'-TACCTAATTATGGGTGCACAATTC-3', reverse: 5'-TCCATTTTGCATAATATGCCACTC-3'. The PCR profile used was 35 cycles with denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. Primers used for bovine c-Met corresponding to the cloned sequences (+3140 to +3400) were as follows—forward: 5'-CCCAACTACAGAATGGTTTCCC-3', reverse: 5'-ATCAGACTGCTCGGCCCAATTA-3'. The PCR profile of 35 cycles was used with denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $58^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  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  $\beta$ -casein (GenBank accession number NM181008; +120 to +672; forward: 5'-CTCAATGTACCTGGTGAGAT-3' and reverse: 5'-AGGCCTGAATGGGCATATCTCT-3') mRNAs were also analyzed.

## 2.3. Immunohistochemistry

Cryostat sections of  $4\ \mu\text{m}$  in thickness were prepared and mounted on glass slides pre-coated 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 h. 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%  $\text{H}_2\text{O}_2$ . The sections were counterstained with hematoxylin.

### 3. 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 2492 nucleotides and contained an open reading frame of 2190 bp encoding 730 amino acids. The cloned bovine c-Met cDNA consisted of 4823 nucleotides with an open reading frame of 4152 bp encoding 1384 amino acids (Supplementary data, 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 (Supplementary data, 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

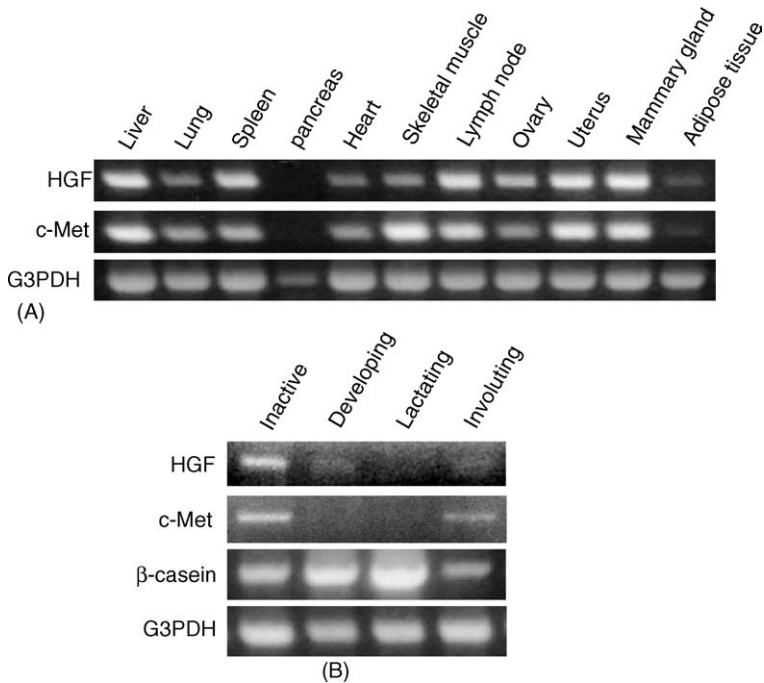
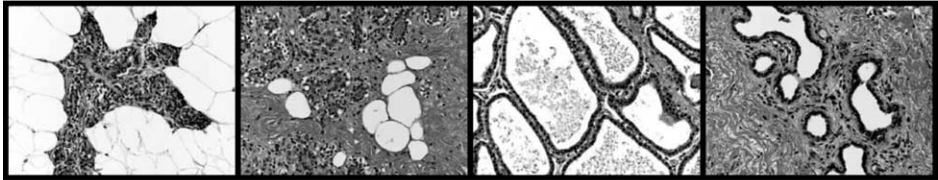


Fig. 1. Expression of HGF and c-Met mRNAs in the bovine tissues. (A) Total RNA (2  $\mu$ g) extracted from various tissues from a non-pregnant cow were subjected to RT-PCR analysis. Shown is a representative result of three cows. (B) Total RNA extracted from the mammary glands at different stages of development under hormonally induced lactation of cows were subjected to RT-PCR analysis. Shown are representative results of each stage of mammary gland development.

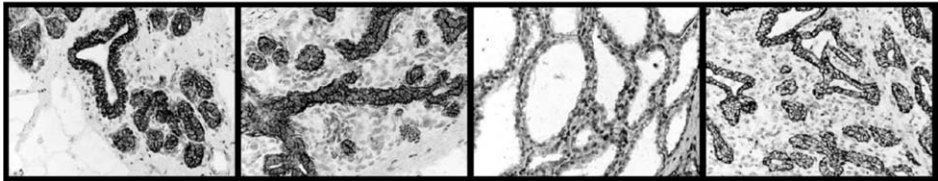
status of which was confirmed by the induction of  $\beta$ -casein mRNA (Fig. 1B). HGF mRNA was detected only in the inactive stage and not in the developing, lactating, and involuting stages. c-Met mRNA was detected in the inactive and involuting stages, but not in the developing and lactating stages.

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 detected a single protein with a molecular weight of 140 kDa in the membrane fractions of lactating mammary gland and cultured mammary epithelial cells, but not of mammary gland from a calf (Supplementary data, Fig. S3). Conversely, HGF immunoreactivity was not detected using anti-human HGF antibody (data not shown).

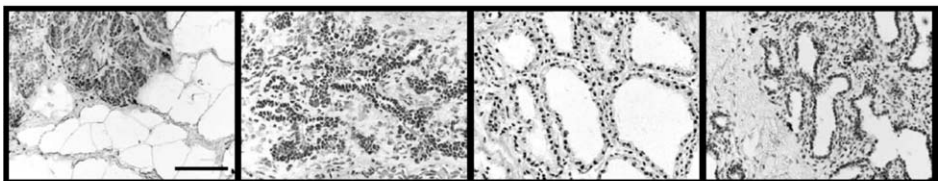
#### HE staining



#### Anti-c-Met antibody



#### Normal Serum



Inactive

Developing

Lactating

Involuting

Fig. 2. Immunohistochemical localization of c-Met in the bovine mammary gland. Cryostat sections ( $4\ \mu\text{m}$ ) 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:  $100\ \mu\text{m}$ .

#### 4. 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 amino acid sequences of bovine HGF and c-Met mRNAs indicated that motifs and amino acids that are important for the maintenance of characteristic structural and functional features of the proteins were conserved (Supplementary data, 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-17 $\beta$  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 140 kDa protein in cultured mammary epithelial cells. Moreover, c-Met protein on mammary epithelial cells, but not myoepithelial 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 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 the 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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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.domaniend.2005.08.005](https://doi.org/10.1016/j.domaniend.2005.08.005).

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