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## Leptin inhibits hepatocyte growth factor-induced ductal morphogenesis of bovine mammary epithelial cells

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### Abstract

We examined the effect of stroma-derived factors, hepatocyte growth factor (HGF) and leptin, on morphological differentiation of bovine mammary epithelial cells (BMEC) in collagen gel three-dimensional culture *in vitro*. BMEC treated with HGF, but not leptin, formed duct-like organoids. The formation of organoids by HGF was enhanced by treatment with a mixture of insulin, cortisol and prolactin, while BMEC treated with the mixture alone did not produce the organoid. In contrast, the formation of organoids by HGF was dose-dependently inhibited by simultaneous addition of leptin, regardless of the presence or absence of the hormone mixture. These results suggest that stroma-derived factors intricately regulate mammary epithelial morphogenesis.

Key Words : adipocyte, mammary gland, obesity

Development of mammary gland occurs during embryonic stages, and also in postnatal life, the latter mainly depends on ovarian steroids such as estrogen and progesterone<sup>8,17,21)</sup>. Recent evidence indicates that these

hormones influence both mammary epithelial and stromal mesenchymal cells, resulting in the regulation of mammary gland development through reciprocal epithelial-stromal interactions<sup>8,17,21)</sup>. The absence of stromal adipo-

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cytes causes disruption of the interactions and subsequently prevents branching morphogenesis, but alveoli formation occurs<sup>4</sup>. Several local factors such as insulin-like growth factor (IGF)-1 and hepatocyte growth factor (HGF) produced from stroma are possible mediators of the interactions that induce branching morphogenesis<sup>11,17,24</sup>.

It was reported that obese rats often lose their litters in the postpartum periods to primary lactation failure<sup>14</sup>. Moreover, diet-induced obese mice also exhibit reduced conception rates, increased peripartum pup mortality, and impaired lactogenesis, which is accompanied by marked abnormalities in alveolar development and reduction in branching frequency of the mammary gland<sup>6</sup>. As obesity is characterized by a state of mild inflammation due to the rise of inflammation-related adipocytokine release<sup>19</sup>, it is likely that adipocytes in obesity produce some inhibitory factor(s) that prevent mammary gland development.

Leptin is one of the major adipocytokine and increases in obesity<sup>7</sup>. Mutation of leptin (*ob*) gene leads to profound obese phenotype of *ob/ob* mice with hyperphagia, reduced energy expenditure and impaired reproductive and endocrine functions. In addition, *ob/ob* mice have minimal epithelial development in the mature mammary gland compared with their lean counterparts<sup>3,10,13</sup>. Administration of recombinant leptin to *ob/ob* mice reduces food intake, produces weight loss and improves reproduction and endocrine status<sup>3,13</sup>. However, *ob/ob* mice treated with leptin fail to lactate<sup>3,13</sup>, leading to a suggestion that the deficiency in lactation may not be directly related to leptin.

We, however, hypothesized that leptin, under certain conditions, acts as an inhibitory factor of mammary gland development. If so, this assumption might explain the reason

why deficiency in lactation in *ob/ob* mice was not rescued by leptin administration and also the reason why abnormal mammary gland development occurred in non-genetically obese animals. To test the hypothesis, we examined the effect of leptin on HGF-induced morphological differentiation of mammary epithelial cells *in vitro*.

## Materials and Methods

### Isolation of bovine mammary epithelial cells (BMEC)

Experimental procedures and care of animals were in accordance with the Guidelines of the Animal Care and Use of Hokkaido University, Japan, and the study was approved by the Committee for the Care and Use of Laboratory Animals in the Graduate School of Veterinary Medicine, Hokkaido University.

BMEC were prepared as described previously<sup>20</sup>. Briefly, mammary gland tissue was obtained from 28 month-old cow (247 days of pregnancy), minced, washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>- free Hank's balanced salt solution, and further digested with a solution containing collagenase (400 units/ml, Wako Pure Chemical Co., Osaka, Japan), elastase (2.8 units/ml, Wako), hyaluronidase (600 units/ml, Sigma-Aldrich, Inc., St. Louis, MO, USA),  $\alpha$ -chymotrypsin (100 units/ml, Worthington Biochemical Co., Freehold, NJ, USA), soybean trypsin inhibitor (10  $\mu$ g/ml, Sigma), deoxyribonuclease I (10 units/ml, Wako) and bovine serum albumin (BSA fraction V, 20 mg/ml, Sigma). The digest was passed through a stainless steel filter (50  $\mu$ m mesh) and then subjected to Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) density gradient centrifugation to enrich epithelial cells. The purity of cells was assessed by detecting cytokeratin, a marker of epithelium, and over 95% of the purified cells were stained with an anti-cytokeratin antibody, but not with an

anti-vimentin antibody that stains stromal cells (data not shown). BMEC were stored in liquid nitrogen until use.

### Three-dimensional culture of BMEC in collagen gel

BMEC were cultured in collagen gels. In brief, 24-well plates (Becton Dickinson, Tokyo, Japan) were first incubated with a mixture of 0.4 ml collagen (1.5 mg/ml Cellmatrix type I-A, Nitta Gelatin, Tokyo, Japan), 0.05 ml 7.5% sodium hydrogen carbonate and 0.05 ml 10-times concentrated basal medium [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1 : 1) (Sigma), BSA (2 mg/ml), penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (10 µg/ml), amphotericin B (250 ng/ml, Fungizone, Invitrogen, Carlsbad, CA, USA)] and left until collagen had gelled as a base. Subsequently, BMEC ( $5 \times 10^4$  cells) in 0.5 ml of the collagen mixture was added onto the base. After the second collagen solution containing the cells had gelled, 0.5 ml of the basal medium supplemented with holotransferrin (10 µg/ml, Invitrogen), insulin (10 µg/ml, Sigma), cortisol (5 µg/ml, Sigma), epidermal growth factor (10 ng/ml, Wako) and cholera toxin (10 ng/ml, Wako) was overlaid. One week after the onset of the culture, the collagen gel was digested with M199 medium (Sigma) containing 1% collagenase and 0.1% soybean trypsin inhibitor, and then with trypsin-EDTA (Invitrogen), to recover the BMEC.

The cells were cultured again in collagen gels for indicated time period with recombinant human HGF (100 ng/ml, Mitsubishi Pharma Co., Tokyo, Japan) and/or a mixture of lactogenic hormones [insulin, cortisol and prolactin (all from Sigma), 5 µg/ml in each] dissolved in the basal medium, as an overlaid solution. The overlaid solution was changed every 2 days. In some experiments, human

leptin (1 or 5 nM, PeproTechEC Ltd., London, UK) was included in the overlaid solution.

BMEC cultured in collagen gel for 14 days were fixed with 10% formalin in 100 mM phosphate buffer, and at least 25 randomly selected organoids per experimental condition were photographed at the same scale under light transmission inverted photomicroscopy, focusing at the level of the central body of each organoids. The picture was analyzed by using Adobe Photoshop and NIH Image software, and the area of each organoid was determined as number of pixels.

### Statistical Analysis

All values were expressed as means  $\pm$  S.E.M. Statistical comparison was made by analysis of variance, followed by Scheffe's F test.

### Results

BMEC were scattered in a collagen gel after being cultured with the basal medium alone (DMEM/Ham's F-12 containing 2 mg/ml BSA) as an overlaid solution for 14 days (Fig. 1A). In contrast, BMEC was assembled, and formed some ductal-like organoids when being cultured with HGF (Fig. 1B). To examine the effect of leptin on the HGF-induced morphological differentiation of BMEC, they were cultured with leptin either in the absence or presence of HGF. BMEC cultured with leptin alone did not form any organoids (Fig. 1C), whereas BMEC cultured with HGF and leptin did form organoids (Fig. 1D). However, size of each organoid formed by HGF and leptin seemed to be smaller than that of HGF alone (see inset of Figs. 1B and 1D). Quantitative analysis showed that leptin significantly and dose-dependently decreased the size of organoids formed by HGF (Figs. 2A and 2C).

BMEC were scattered in a collagen gel

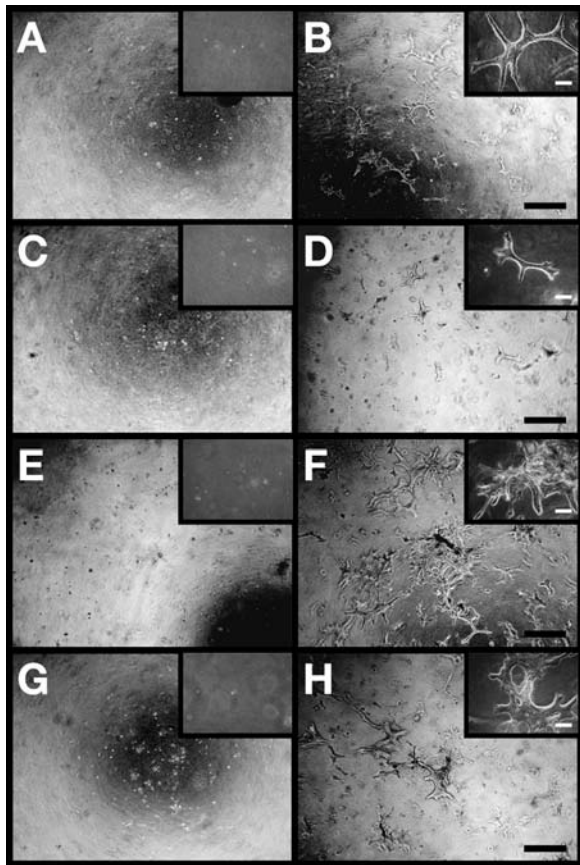


Fig. 1. Morphological differentiation of bovine mammary epithelial cells induced by HGF, leptin and a mixture of insulin, cortisol and prolactin.

BMEC were cultured in collagen gels for 14 days with the basal medium alone (DMEM/Ham's F-12 containing 2 mg/ml BSA) as an overlaid solution (A), or the basal medium containing HGF (B, 100 ng/ml), leptin (C, 5 nM), or both (D). BMEC were also cultured with the basal medium containing a mixture of insulin (5  $\mu$ g/ml), cortisol (5  $\mu$ g/ml) and prolactin (5  $\mu$ g/ml) (E), or that containing HGF (F), leptin (G), or the both (H). Shown are representative photographs at lower and higher magnifications. Black and white scale bars indicate 500  $\mu$ m and 100  $\mu$ m in length, respectively.

culture with the basal medium containing only a mixture of insulin, cortisol and prolactin (lactogenic hormones, Fig. 1E). BMEC cultured with HGF and the lactogenic hormones formed organoids (Fig. 1F) that were well de-

veloped and significantly larger than that of HGF alone ( $528 \pm 35 \times 10^3$  pixels and  $255 \pm 17 \times 10^3$  pixels, respectively,  $p < 0.05$ ) (Fig. 2). BMEC cultured with the lactogenic hormones and leptin were scattered (Fig. 1G), but formed organoids when being cultured with HGF, the lactogenic hormones and leptin simultaneously (Fig. 1H). However, in the presence of leptin, size of organoids formed by HGF and the lactogenic hormones was significantly decreased (Figs. 2B and 2C).

### Discussion

In the present study, we have demonstrated that HGF, either in the presence or absence of the lactogenic hormones, induces morphological differentiation of BMEC, and also that leptin is an inhibitory factor for HGF-induced morphological differentiation of BMEC.

Although there are some species differences<sup>9</sup>, mammary gland development during pregnancy basically follows a similar pattern. The mammary gland in the resting state consists of minimum branching ducts embedded in stromal adipose tissue. Following the onset of pregnancy, the development of the mammary gland begins with the ductal branching and then alveolar morphogenesis of epithelial cells. Subsequently, alveolar epithelial cells differentiate functionally to produce and secrete milk during lactation<sup>8,17,21</sup>. It is noteworthy that leptin and leptin receptor are expressed at the greatest during mid-pregnancy in ovine mammary gland, when active growth of mammary gland is initiated<sup>1,12</sup>. In addition, HGF is shown to promote branching of the ductal trees, but inhibit the production of milk proteins<sup>23</sup>. Collectively, in the process of mammary gland development, the emergence of leptin suppression on HGF-induced morphogenesis may facilitate a stage switching from ductal formation to alveolar develop-

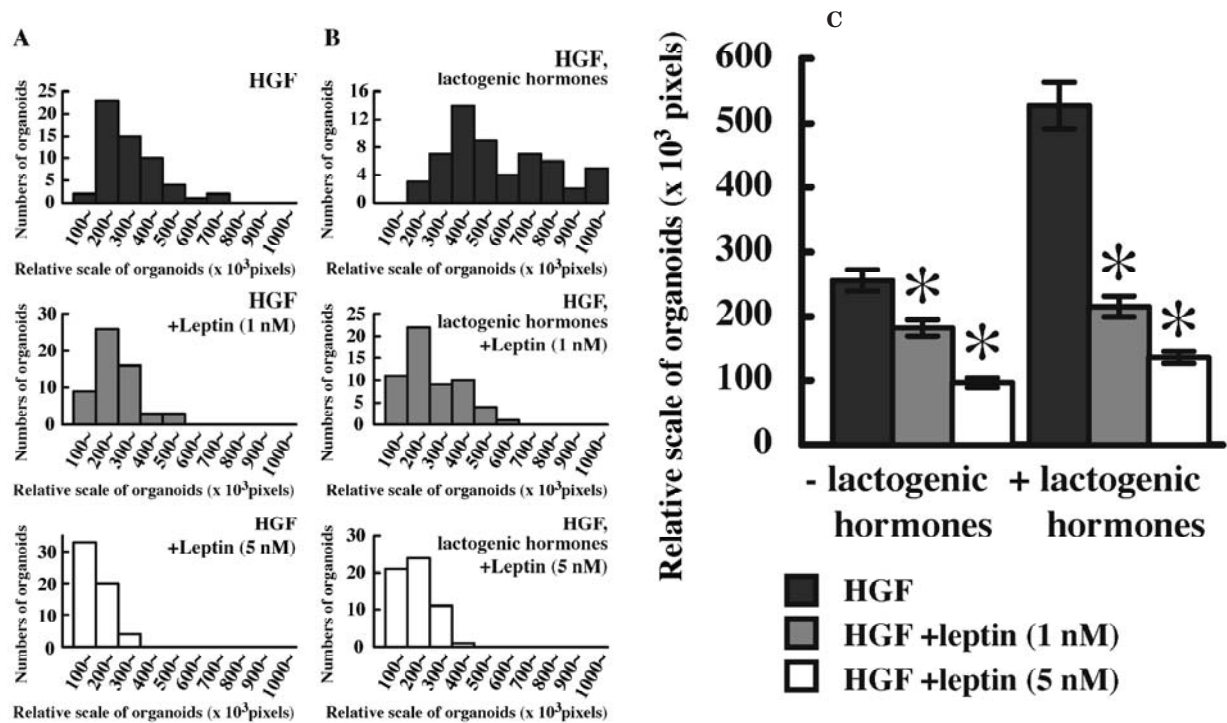


Fig. 2. Quantitative analysis of the morphological differentiation of bovine mammary epithelial cells.

A. BMEC were cultured in collagen gels for 14 days with the basal medium alone (DMEM/Ham's F-12 containing 2 mg/ml BSA) containing HGF (100 ng/ml) alone (upper panel), or that containing 1 nM or 5 nM leptin (middle and lower panels, respectively). B. BMEC were cultured with the basal medium containing HGF and a mixture of insulin (5  $\mu$ g/ml), cortisol (5  $\mu$ g/ml) and prolactin (5  $\mu$ g/ml) (lactogenic hormones, upper panel), or that containing 1 nM or 5 nM leptin (middle and lower panels, respectively). Size of each organoid as observed in Fig. 1 was quantified and the results of three separate experiments were summarized as histograms. C. Average size of the organoid formed in the different culture conditions were compared statistically. \*,  $p < 0.05$  vs. HGF either in the presence or absence of lactogenic hormones.

ment.

The *ob/ob* mice suffer several abnormalities in reproduction and endocrine functions, that could be rescued by leptin administration, except lactation<sup>3,10,13</sup>. Since the present results suggest that leptin inhibits ductal formation of mouse mammary epithelial cells, replacement of leptin in *ob/ob* mice probably happens to inhibit lactation by direct inhibitory effect on mammary epithelial cells.

In massive obesity, leptin is present in the circulation at extremely high concentrations. Thus, it is likely that the super physiological concentrations of leptin derived from both blood and local adipocytes, inhibit initial

or early ductal formation and consequent alveolar development. The assumption is supported, at least in part, by recent findings that diet-induced obese mice show marked abnormalities in alveolar development and reduction in branching frequency of the mammary gland<sup>6</sup> and the fact that obesity impairs lactogenesis in animal models and livestock animals, and possibly in human<sup>2,6,14,15</sup>.

The mechanism(s) by which leptin affected HGF-induced BMEC morphogenesis is currently unknown, but likely attenuates the HGF-induced BMEC proliferation. However, there are contradictory reports concerning the effects of leptin on mammary epithelial cell

growth. Silva et al. reported the suppression of IGF-I-induced proliferation<sup>16)</sup>, while Thorn et al. showed no effect on BMEC proliferation<sup>18)</sup>. Furthermore, leptin is shown to promote the growth of breast cancer cells<sup>5,10)</sup>. Therefore, further studies on the leptin effects on mammary epithelial cells are warranted, and our investigations on the cellular and intracellular mechanisms of the leptin actions are ongoing.

In summary, the present study showed that HGF and leptin, both stroma-derived factors<sup>1,11,24)</sup>, intricately regulated morphological differentiation, especially branching ducts formation of mammary epithelial cells, which express both c-Met (HGF receptor) and OBRb (leptin receptor)<sup>12,22)</sup>.

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