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Cell Density-Dependent Regulation of Hepatic Development by a gp130-Independent Pathway

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We previously demonstrated that oncostatin M (OSM) promotes hepatic development in concert with glucocorticoid. The livers from mice deficient for gp130, a signaling subunit of the OSM receptor, displayed reduced expression of hepatic differentiation marker and defective glycogenic function. However, these phenotypes were not completely abolished in gp130$^{-/-}$ mice, suggesting that there is an alternative pathway regulating hepatic development *in vivo*. To test this possibility, we cultured gp130$^{-/-}$ fetal hepatic cells and investigated a signal that induces hepatic differentiation. When hepatocytes were forced to interact with each other by inoculating cells at high densities, hepatic differentiation was induced even in the absence of gp130. Moreover, cells stimulated with OSM and/or cultured at a high density possess many other metabolic functions. These observations suggest that fetal hepatic cells acquire multiple characteristics of differentiated hepatocytes in response to the signals generated by cell–cell contacts as well as by OSM.

Key Words: **hepatic development; cell density; oncostatin M; gp130; STAT3; knockout mouse; metabolic function.**

Adult hepatocytes are terminally differentiated cells that display numerous liver-specific functions. These functions are developmentally regulated; for example, albumin is expressed from the early stage of liver development (1), while enzymes required for metabolic functions are mostly absent until birth (2–9). After the terminal differentiation, hepatocytes express numerous liver functions and do not proliferate except under pathologic states, such as liver regeneration and tumorigenesis (10, 11).

Key factors controlling liver metabolic functions are hormones and cytokines. Two major peptide hormones, insulin and glucagon, mainly regulate the balance between catabolic and anabolic activities of the adult liver. These hormones also regulate expression of hepatic differentiation markers such as tyrosine amino transferase (TAT), glucose 6-phosphatase (G6Pase), tryptophan oxygenase, and serine dehydratase (12, 13). In addition, production of acute phase proteins is another important function of the liver and is regulated by several inflammatory cytokines, e.g., IL-1, IL-6, and TNFα (14–18).

Not only soluble mediators but also cell–cell contact control the functions of the adult liver. It was shown that proliferative and metabolic activities of primary hepatocytes are modulated by cell density (19). At a high-cell density that allows direct cell–cell interaction, cells tend to exhibit differentiated functions and do not proliferate even in the presence of a mitogen. In contrast, cells at a lower density are less functional and are more responsive to mitogenic stimulation. Direct cell–cell interaction is thus suggested to play a key role in the transition between G1 and G0 phases of the cell cycle (20). Recently, the importance of cell density in fetal hepatocytes was also reported (21), suggesting that cell–cell interaction regulates embryonic liver development. Although the molecular basis as to how cell density affects hepatic functions is unknown, cell adhesion through adherens junctions, tight junctions, and gap junctions is likely to be involved, since intracellular signals are generated through these junctions (22–24).

We recently reported that oncostatin M (OSM), an IL-6-related cytokine produced by hematopoietic cells, stimulates development of fetal hepatic cells in pri-

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mary culture (25, 26). We also showed that knockout mice deficient for gp130, a key signaling component of the OSM receptor, showed reduced expression of hepatic differentiation markers and defective glycogenic function (25). However, in gp130$^{-/-}$ livers, expression of differentiation markers was not completely abolished and there remained some cells positive for glycogen near the periphery of the liver lobules. These observations suggest that there is an alternative pathway that stimulates hepatic development in the absence of gp130.

In this study, we performed single embryo culture of fetal hepatic cells derived from gp130$^{-/-}$ mice (E14.5) and investigated the condition that activates the differentiation program of the liver in the gp130$^{-/-}$ background. We found that forced interaction between cells leads to upregulation of hepatic differentiation markers and of the glycogenic activity in gp130$^{-/-}$ cells. Studies using normal embryos show that both OSM and cell–cell contact induce multiple characteristics of mature hepatocytes, e.g., ammonia clearance, albumin production, and the gene expression profile. Our results suggest that not only signals induced by soluble factors but also signals generated through cell–cell contact contribute to functional maturation of fetal hepatic cells.

MATERIALS AND METHODS

Cell culture and growth media. Fetal hepatic cells were isolated from E14.5 embryonic liver tissues of gp130$^{-/-}$ (ICR) (27) mice or normal C57Br/6CrSlc mice (Nihon SLC, Hamamatsu, Japan) and cultured as described previously (25). Briefly, minced liver tissues were enzymatically dissociated with Liver Digest Medium (Gibco BRL). Contaminating erythrocytes were hemolyzed with hypotonic buffer. Cells were plated onto 0.1% gelatin-coated tissue-culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) at 2×10^4 cells/cm^2 unless otherwise indicated. In some cases, cells were inoculated at 6×10^4 cells/cm^2 (high) or 1×10^5 cells/cm^2 (extra high). Four hours later, nonadherent hematopoietic cells and cell debris were washed out with phosphate-buffered saline (PBS). Culture media and cytokines were changed every 2 days.

Analysis of mRNA expression by Northern blotting. Ten μg of total RNA of each sample extracted by AGPC methods was separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde and transferred to a positively charged nylon membrane. Membranes were hybridized with digoxigenin (DIG)-labeled cDNA probes and further incubated with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim). Blots were developed with the CDP-star reagent (New England Biolabs, Beverly, MA).

Analysis of phosphorylation of STAT3 by Western blotting. Stimulated or nonstimulated cells were washed three times with PBS and lysed with lysis buffer (50 mM Tris–HCl [pH 7.4], 1% Triton X-100, 0.5 mM Na_3VO_4, 5 mM EDTA, 1 mM leupeptin, and 0.1 mM Pefablock [Sigma Chemical]). Lysates were incubated for 1 h at 4°C with gentle agitation and cleared by centrifugation at 10,000*g* for 15 min. Equal amounts of protein samples denatured with Lemmli's solution were subjected to 7.5% SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membrane was incubated with either anti-phospho-STAT3 antibody (New England Biolabs) or anti-STAT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a second antibody conjugated with horseradish peroxidase. The immune complex was visualized by the ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Measurements of ammonia and albumin in culture media. To examine cellular activity of ammonia detoxification, fetal hepatic cells were loaded with 2 mM NH_4Cl at day 4 or 7 after plating and further incubated for 0–24 h. Concentrations of NH_4Cl remaining in culture media were measured at various time points by the modified indophenol method using a commercial kit (Ammonia-test Wako; Wako Pure Chemical Industries). For analysis of albumin secretion, cells at day 7 were washed with PBS and incubated with culture media without serum for 24 h prior to assays. The albumin protein level was determined by the brom-cresol-green method (Albumin-test Wako; Wako Pure Chemical Industries). The activities of ammonia clearance and albumin secretion were determined by dividing total amounts of cleared ammonia and secreted albumin by the incubation time and the cell number used for assays. Data represent mean values ± SD of at least triplicate experiments.

Analysis of glycogen accumulation. Cells cultured with or without OSM for 7 days were fixed with 20% formaldehyde and stained with the PAS staining solution (Muto Pure Chemicals, Tokyo, Japan) according to the standard protocol.

RESULTS

Induction of multiple differentiation markers and glycogenic activity in gp130$^{-/-}$ fetal hepatic cells. Since fetal hepatocytes proliferate autonomously and are not tightly interacting with each other, we usually inoculate hepatic cells at the low density (2×10^4 cells/cm^2) at the beginning of cultures. As we showed previously, cells under this condition adequately reconstitute characteristics of fetal hepatocytes *in vivo* as judged by gene expression profile, growth property, and ability to support hematopoiesis (25, 26). However, the cell density has profound effects on liver functions (19, 20), and indeed, direct cell–cell contact *in vivo* increases during the late-fetal development. We thus reasoned that signals generated through cell–cell interaction could modulate differentiative responses of embryonic hepatocytes in the gp130$^{-/-}$ background. Therefore, we cultured gp130$^{-/-}$ fetal hepatic cells (E14.5) at two different cell densities and examined for expression of various differentiation markers (Fig. 1) and glycogen accumulation (Fig. 2). In wild type cells cultured at the low density (2×10^4 cells/cm^2), OSM induced expression of all marker genes tested here. When cells were cultured at the high density (6×10^4 cells/cm^2) (Fig. 1, lane 5), these genes were expressed even in the absence of OSM and the levels of expression were further augmented by the addition of OSM (Fig. 1, compare lanes 5 and 7). In contrast, gp130$^{-/-}$ cells failed to respond to OSM at both densities, confirming that OSM transduces differentiation signal through gp130. Interestingly, mRNAs for TAT and carbamoylphosphate synthetase-1 (CPS-1) were clearly detected in gp130$^{-/-}$ cells at the high density, although there was no additive effect of OSM. Likewise, glycogenic activity was up-regulated in gp130$^{-/-}$ cells at the high density. Thus, cell density-mediated induction of hepatic development occurs in the absence of gp130.

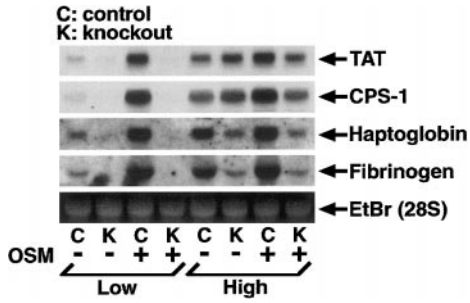

FIG. 1. Induction of hepatic differentiation in gp130$^{-/-}$-derived hepatic cells. Hepatic cells isolated from gp130$^{-/-}$ mice were inoculated either at 2×10^4 cells/cm^2 (low) or at 6×10^4 cells/cm^2 (high) and cultured in the presence of dexamethasone with or without OSM for 7 days. Total cellular RNAs were extracted and analyzed by Northern blot for expression of hepatic differentiation markers. Data show that hepatic differentiation markers were induced in gp130$^{-/-}$ cells at the high density.

However, induction of haptoglobin and fibrinogen was minimal in gp130$^{-/-}$ cells at the high density. Since these two genes belong to acute phase proteins, it is possible that they are more strictly dependent upon cytokine-induced signals. Alternatively, a certain intracellular signaling molecule required for induction of these genes at the high density might be lost during embryonic development in gp130$^{-/-}$ cells.

Cell density-dependent expression of differentiation markers. The results described above clearly suggest that signals generated through cell–cell interaction

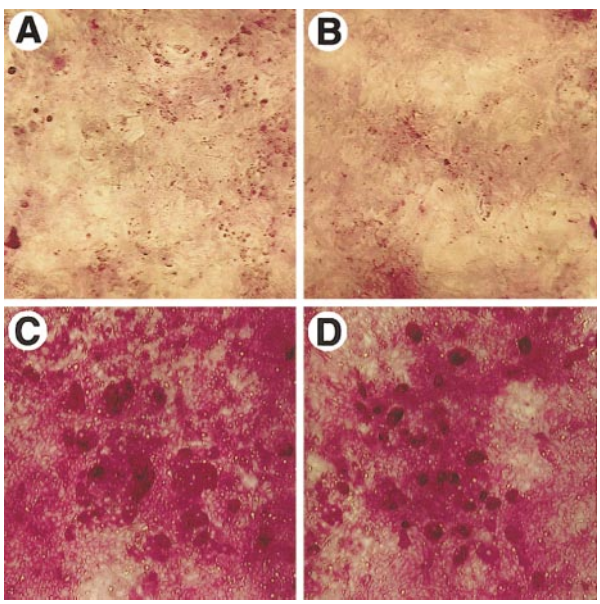

FIG. 2. Induction of glycogenic activity in gp130$^{-/-}$-derived hepatic cells. Hepatic cells were seeded at two densities, i.e., 2×10^4 cells/cm^2 (low: A, B) and 6×10^4 cells/cm^2 (high: C, D) and cultured for 7 days in the presence (B, D) or absence of OSM (A, C). Intracellular glycogen was detected by the *in vitro* PAS staining method.

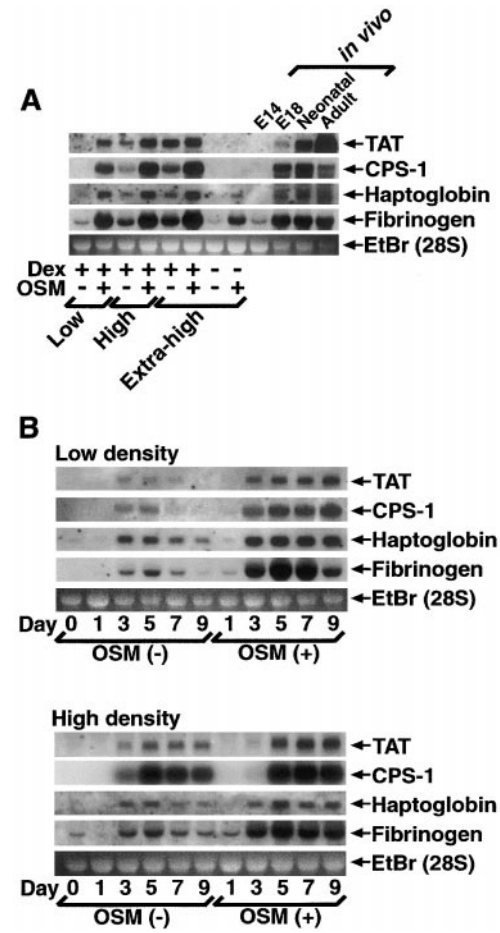

FIG. 3. Induction of hepatic differentiation markers by cell–cell contact and by OSM. (A) Fetal hepatic cells were inoculated at 2×10^4 cells/cm^2 (low), 6×10^4 cells/cm^2 (high), or 1×10^5 cells/cm^2 (extra-high) and cultured with or without OSM (10 ng/ml) in the presence of Dex for 7 days. Ten μg of total RNA from each sample was analyzed for expression of TAT, CPS-1, haptoglobin, or fibrinogen mRNA by Northern blotting. Note that differentiation markers were induced in the absence of OSM at higher densities. (B) Cells were cultured with Dex alone (10^{-7} M) or Dex plus OSM (10 ng/ml) for 1, 3, 5, 7, or 9 days at low or high density. Ten μg of total RNA samples were tested for expression of hepatic differentiation markers.

contribute to hepatic maturation in a gp130-independent manner. We next examined more precisely how much the cell density affects various parameters of hepatic differentiation in fetal hepatocytes with a normal genetic background. First, induction of marker genes was investigated using three different cell densities. As shown in Fig. 3A, the cells expressed differentiation markers in the absence of OSM when they were cultured at the high density, and the level of expression was further augmented by OSM. At five times higher density (extra-high), the levels of gene expression were nearly equivalent to those in OSM-stimulated cells at the low density. These results indicate that forced interaction between hepatic cells re-

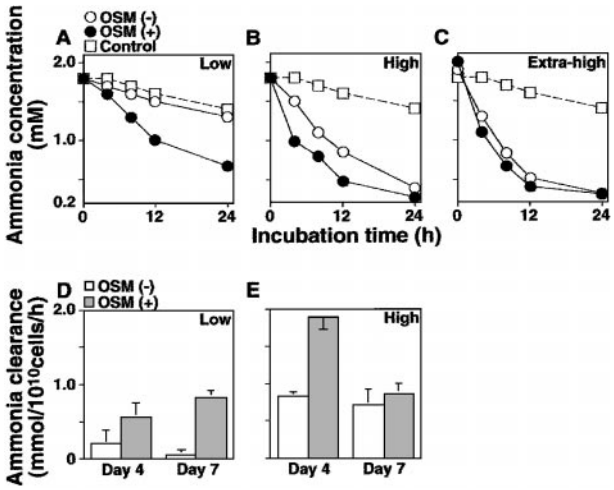

FIG. 4. Stimulation of ammonia clearance from culture media by OSM and in high-density cultures. Cells cultured under various conditions were examined for their potential to eliminate ammonia from culture media. On day 7 (A–E) or day 4 (D, E) after plating, the culture medium was changed to fresh medium containing 2 mM NH_4Cl and the ammonia concentration remaining in the culture media was monitored for next 24 h. (A–C) Kinetic analysis of ammonia concentration remaining in culture media. (D, E) Specific activity of ammonia clearance of days 4 and 7. Data represent mean values ± SD of at least triplicate experiments.

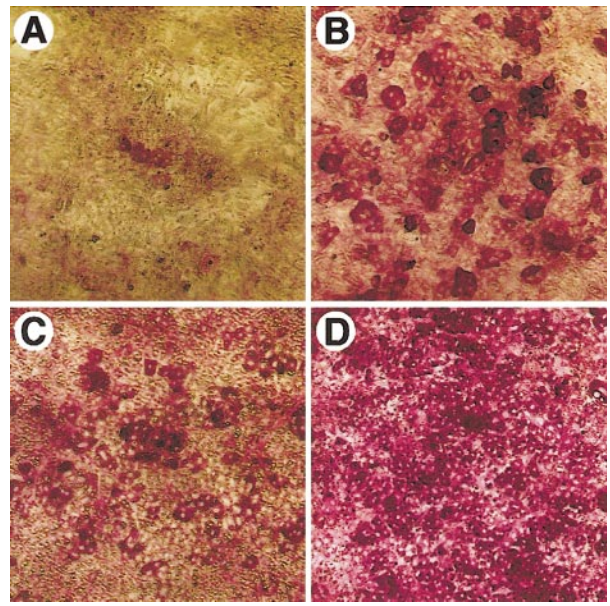

FIG. 6. Regulation of glycogenesis by OSM and by cell density. Cells were cultured for 7 days in the presence (B and D) or absence (A and C) of OSM at two different cell densities (A and B, low; C and D, high). Cells were then stained with the PAS solution to detect intracellular glycogen.

duces the OSM requirement for induction of differentiation markers. Timecourse analyses shown in Fig. 3B demonstrate that both OSM- and cell density-induced expression of marker genes were detected from day 3 and reached the maximal level on days 7–9. Thus, the kinetics of gene induction by OSM and by cell density were similar, although different intracellular mechanisms is likely to mediate these two differentiation signals.

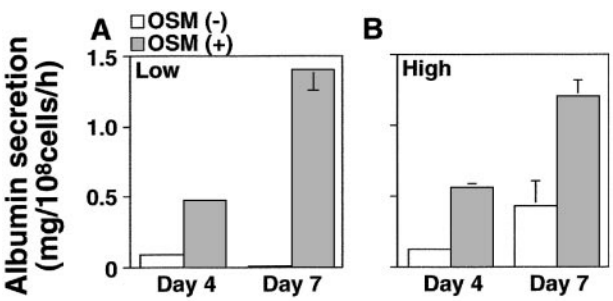

FIG. 5. Stimulation of albumin secretion by OSM and in high-density cultures. Effects of OSM administration and high cell density on albumin secretion were tested. Cells were inoculated at low or high density and cultured for 4 or 7 days in the presence or absence of OSM. Culture media were changed to serum-free at 12 h before assays to exclude serum-derived albumin. Cells were incubated for another 24 h and amounts of albumin secreted into culture media were measured by the brom-cresol-green method. Albumin secretion activity is shown as the amount of secreted albumin(mg)/cell/h. Data represent mean values ± SD of at least triplicate experiments.

Ammonia clearance by fetal hepatic cells. We next examined metabolic activities of fetal hepatic cells stimulated with OSM and/or high density. Clearance of ammonia from the blood circulation is an important function of the adult liver. In hepatocytes, ammonia is converted to urea through the ornithin cycle or to amino acids such as glutamine and glutamic acid. Loss of ammonia clearance *in vivo* immediately results in severe damages particularly to the central nervous system. As this function is known to be established before birth, we tested whether fetal hepatic cells are able to clear ammonia from culture media (Fig. 4). At the low density, 2 mM of input ammonia rapidly declined to less than 0.5 mM within a day in the presence of OSM and Dex, whereas no significant change of the ammonia concentration was observed in the culture without OSM. On the other hand, cells at higher densities showed more efficient clearance of ammonia and the difference between plus and minus OSM was decreased by increasing the cell density. At the extra-high density, stimulation with OSM had no additional effect on ammonia clearance, while the high-density culture exhibited an intermediate activity, correlating with the induction of differentiation markers (Fig. 3A). Figures 4D and 4E show the specific activity of ammonia clearance (mmol/10^{10}cells/h) calculated from the above experiments, demonstrating that both OSM and high-cell density upregulate the activity of ammonia clearance per cells.

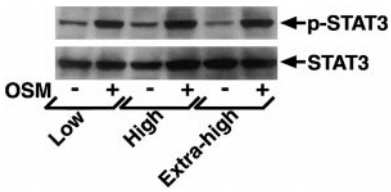

FIG. 7. Lack of tyrosine phosphorylation of STAT3 in high-density cultures. Fetal hepatic cells were inoculated at low, high, or extra-high density and incubated for 7 days with or without OSM in the presence of Dex. Total cell lysates were separated on SDS–PAGE, transferred onto a nitrocellulose membrane, and probed with either anti-phosphoSTAT3 (Tyr 705; upper) or anti-STAT3 antibody (lower). Phosphorylation of STAT3 at Tyr 705 is believed to correlate with STAT3 activation. Note that high-density cultures did not lead to tyrosine phosphorylation of STAT3.

Regulation of albumin synthesis and glycogenesis by OSM and cell density. To further characterize fetal hepatic cells induced to differentiate *in vitro*, we investigated other functional activities of these cells. Synthesis and secretion of albumin are typical liver-specific functions, both of which are known to be regulated by many extracellular stimuli (11, 28–30). We examined whether OSM and the cell density affect the albumin secretion (Fig. 5). At the low density, hepatocytes did not secrete albumin but the addition of OSM stimulated albumin secretion more than 100 times over the control level. Inoculation at the high-cell density induced cells to secrete albumin in the absence of OSM, indicating that both OSM and cell density regulate albumin secretion *in vitro*.

We next analyzed accumulation of intracellular glycogen, since it is known that the glycogenic activity of the liver increases at around birth during development. Cells stimulated with Dex alone accumulated only a small amount of glycogen (Fig. 6A) and OSM did not activate glycogenesis in the absence of Dex (data not shown). In contrast, a number of PAS-positive cells appeared in the culture when they were stimulated with both OSM and Dex (Fig. 6B), consistent with our previous observation (25). Expectedly, at the high density (Fig. 6C), PAS-positive cells were found even in the absence of OSM and the number of PAS-positive cells was further increased by the addition of OSM (Fig. 5D). These results indicate that both OSM and cell density modulate glycogenic activity of fetal hepatic cells as well.

Hepatic development induced by cell density is independent of STAT3 activation. We recently found that OSM activates the STAT3 pathway in fetal hepatic cells and induction of differentiated properties of cells was interrupted by retrovirus-mediated expression of a dominant negative form of STAT3 (Ito *et al.*, submitted) into cells. We therefore asked whether STAT3 is activated in cells at high-cell densities in the absence of OSM. As shown in Fig. 7, neither high nor extra-high density culture resulted in phosphorylation of STAT3, excluding the possibility that hepatic maturation induced in high-density cultures involves STAT3 activation.

DISCUSSION

We demonstrated in this study that there is a gp130-independent pathway that promotes hepatic development. Such a signal is probably generated through cell–cell interaction, since seeding cells at a high density resulted in upregulation of multiple phenotypes of the differentiated liver even in gp130$^{-/-}$ fetal hepatic cells. This could be one reason why gp130 deficiency did not completely abolish expression of a hepatic differentiation marker (i.e., TAT) and glycogenic activity in the perinatal livers (25). Thus, single embryo culture using knockout mice with embryonic-lethal phenotypes is an useful system to analyze liver development, particularly in cases where the lethality is caused by defects in other tissues. Since fetal hepatic cells are readily infected with retrovirus vectors (Ito *et al.*, submitted), genetic complementation of defective phenotypes in knockout mice will provide a means to understand the mechanism of liver development.

Cells stimulated with OSM and/or high density strikingly exhibited various metabolic functions of the mature liver, including gene expression, glycogenic activity, albumin production, and detoxification (ammonia clearance). The effect of cell density is dose-dependent and potentially eliminates the requirement for OSM during hepatic maturation *in vitro*. At the E14.5 stage, contacts between hepatic cells are not fully established and in addition there are a large number of hematopoietic cells which may function as a "buffer" for the direct cell–cell contact between hepatocytes. Homophilic interactions between hepatocytes *in vivo* increase near birth and multiple types of cell adhesion structures are formed. In this context, the extra-high density, where OSM has no additive effect, might be artificial because hepatic cells interact with each other from the beginning of culture. We thus consider that the low to high density is closer to the physiological condition. This also agrees with our previous observation that hepatic maturation *in vivo* requires the signal from the gp130 (25). Probably, the OSM/OSMR system and direct cell–cell interaction coordinately control the process of hepatic development.

An important question to be answered is how cell–cell contact induces hepatic maturation in the absence of OSM stimulation. Our recent results have shown that STAT3 activation is essential for the OSM-induced hepatic differentiation *in vitro* (Ito *et al.*, submitted). However, hepatocytes even at the extra-high density did not induce apparent activation of STAT3 (Fig. 7), even though the maximal differentiation was induced in this condition. These results suggest that

high-density cultures activate a signaling pathway distinct from the STAT3 pathway. Alternatively, a signal induced by the cell–cell contact might have overridden the downstream of the STAT3 signaling pathway. We recently found that an inhibitor for protein tyrosine phosphatases (PTPs) interferes both OSM-induced and cell density-mediated hepatic differentiation. Moreover, several intracellular proteins are dephosphorylated in cells induced to differentiate *in vitro.* PTPs have been implicated in cellular differentiation in many other cell types (31, 32) and a subset of PTPs were shown to mediate homophilic interactions of cells (33, 34). It is therefore an interesting possibility that PTP-dependent dephosphorylation of an intracellular molecule(s) mediates signals from both OSM and cell density in fetal hepatic cells.

Finally, our results suggest that fetal hepatic cells have a potential for clinical application. Until now, primary adult hepatocytes and transformed hepatomas have been used for an artificial liver system. However, the systems to control their proliferative and differentiative properties are not fully established yet. On the other hand, fetal hepatic cells proliferate well and are induced to express multiple metabolic functions of the differentiated liver as described in this paper. Therefore, our culture system may provide a novel cellular material for an artificial organ.

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