STAT3 Down-regulates the Expression of Cyclin D during Liver Development*

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As the expression of cyclin D1 is induced during liver regeneration and also in hepatic tumor cells, cyclin D1 is likely to play an important role in the proliferation and transformation of hepatocytes. However, the role of cyclin D1 in liver development remains unknown. Here we show that the expression of D-type cyclins including cyclin D1, D2, and D3 is down-regulated along with liver development. In addition, oncostatin M (OSM), an interleukin-6 family cytokine, down-regulated the expression of cyclin D1 and D2 in a primary culture of fetal hepatocytes in which OSM induces hepatic differentiation. Ectopic expression of receptor mutants defective in the activation of either STAT3 or SHP-2/Ras indicated that the down-regulation of D1 and D2 cyclins by OSM was mediated by STAT3 but not by SHP-2/Ras. Consistently, expression of dominant negative STAT3 but not Ras relieved OSM-induced suppression of cyclin D expression. Activation of STAT3 in fetal hepatocytes of transgenic mice expressing the STAT3-estrogen receptor fusion protein by 4-hydroxytamoxifen resulted in the suppression of cyclin D1 and D2 expression. These results indicate that STAT3 activation is necessary and sufficient for down-regulation of D1 and D2 cyclins in fetal hepatocytes. Furthermore, STAT3-C, a constitutively active form of STAT3, suppressed transcription of the cyclin D1 promoter in fetal hepatocytes, whereas it activated the transcription in hepatic tumor cells, huH7 and HepG2. Thus, STAT3-mediated down-regulation of cyclin D expression is rather specific to fetal hepatocytes that are undergoing maturation processes including a reduction of their proliferation potential.

The cell cycle is tightly regulated by cell cycle regulatory molecules including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. D-type cyclins including D1, D2, and D3 cyclins interact with CDKs (cdk4, cdk6, and cdk2) during the mid- to late-G1 phase and activate CDKs, the activity of which is negatively controlled by CDK inhibitors (p15INK4A, 16INK4B, 18INK4C, p19INK4D, p21WAF1, and p27Kip1) (1, 2). Among D-type cyclins, D1 cyclin is known to be a key player in the regulation of progression through the G1 phase and of the transition from the G1 to S phase (3, 4). The expression of cyclin D1 is rapidly induced by mitogenic signals in the G1 phase and is also increased by gene amplification and by oncogene products such as Ras and Src in various tumor cells (5–9). Conversely, inhibition of cyclin D1 expression leads to growth arrest in tumor cells (10, 11). In addition, it was shown that the expression of cyclin D1 is suppressed by interleukin-6 (IL-6) during differentiation of M1 hematopoietic cells (12). Thus, cyclin D1 is an important regulator for cell proliferation, tumorigenesis, and also differentiation.

IL-6 family cytokines, which include IL-6, IL-11, BSF3, ciliary neurotrophic factor, leukemia inhibitory factor, and oncostatin M (OSM), play important roles in various cells; e.g. IL-6 induces differentiation of many cell types including myeloid cell lines, M1, Y6, and 1A9-M (13–17). Leukemia inhibitory factor is essential for self-renewal of murine embryonic stem cells (18, 19), and OSM induces differentiation of murine fetal hepatocytes (20) and growth arrest of human A375 melanoma cells (21). These IL-6 family cytokines manifest their functions through receptors that consist of a ligand-specific subunit(s) and the common signal transducer, gp130 (22). Binding of a ligand to its cognate receptor induces activation of Janus kinases that are bound to the receptor, leading to phosphorylation of the docking sites in the receptor for SHP-2 and STAT3. Following the recruitment of signaling molecules, SHP-2 transmits the signal to the Ras-MAPK cascade and phosphorylated STAT3 forms a dimer and is translocated to the nucleus where it regulates gene expression by binding to its target sequences (22). Studies using receptor mutants and dominant negative mutants of signaling molecules have demonstrated that STAT3 is a key regulator of IL-6 function, such as macrophage differentiation and acute phase responses (22, 23). In addition, it was shown that IL-6 induces apoptosis in 1A9-M cells (17), whereas it activates anti-apoptotic signals in IL-3-dependent Ba/F3 cells (24, 25). Thus, the intracellular signals activated by IL-6 lead to various outcomes depending on the target cells.

The proliferation and differentiation of hepatocytes are regulated by various external signals, such as hormones, cytokines, extracellular matrix, and cell-cell contacts. Glucocorticoid modulates the proliferation and function of hepatocytes in vivo and in vitro and transforming growth factor-β is a potent inhibitor of hepatic growth (26, 27). IL-6 family cytokines are involved in the development, function, and regeneration of the
liver. For example, IL-6-deficient mice exhibit defects in liver regeneration after partial hepatectomy and CCl4 liver injury (28). While the liver is apparently normal in gp130-deficient mice at the time of birth when mutant mice die, gp130-deficient liver exhibits metabolic defects such as a limited accumulation of glycogen and decreased expression of enzymes for metabolism (20). OSM is a paracrine factor produced by hematopoietic cells in fetal liver and induces differentiation of fetal hepatocytes (20). By using a primary culture of fetal hepatocytes derived from murine embryos at embryonic day 14.5 (E14.5), it was demonstrated that STAT3 and K-Ras mediate the OSM signaling for functional maturation of fetal hepatocytes into neonatal liver cells and morphological changes including the formation of E-cadherin-based adherens junctions, respectively (29, 30).

Differen...tiated hepatocytes in adult liver are quiescent and exhibit various metabolic functions; however, liver injury induces proliferation of hepatocytes at the expense of metabolic functions. Most hepatocellular carcinomas actively proliferate, but they lose many functions of mature hepatocytes, indicating that they are de-differentiated by transformation. Consistently, fetal hepatocytes proliferate vigorously but lack most mature liver functions. Thus, the proliferation and function of hepatocytes are inversely related. Consistent with these observations, evidence is accumulating that the proliferation of hepatocytes is correlated with the expression of D-type cyclins, particularly cyclin D1. The expression of cyclin D1 is up-regulated at the initial phase of liver regeneration induced by partial hepatectomy and CCl4 (31, 32). In addition, amplification of the cyclin D1 gene is found in 13% of hepatocellular carcinomas (33). These observations suggest that the regulation of cyclin D1 expression is critical for the proliferation and differentiation of hepatocytes. However, the regulation of cyclin D expression in liver development remains unexplored.

In this study, we investigated the mechanism underlying the regulation of D-type cyclins during liver development. The expression of D-type cyclins was down-regulated along with liver development in vivo, and OSM suppressed the expression of cyclin D1 and D2 in fetal hepatocytes in vitro. We provide evidence that this negative regulation is specific to fetal hepatocytes and is mediated by STAT3 but not Ras.

**Experimental Procedures**

Primary Culture of Fetal Hepatocytes and Cell Culture—Fetal livers were isolated from E14.5 embryos of C57BL/6CrSlc mice or STAT3ER chimeric mice. Isolated livers were dissociated with a collagenase-based dissociation buffer (liver digest medium, Invitrogen) followed by hemolysis with a hypotonic buffer as described previously (20). Dissociated cells were inoculated at a density of 2 x 10^6 cells/cm² in 0.1% gelatin-coated dishes (Costar) and then cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, minimum Eagle’s medium non-essential amino acid solution (Invitrogen), insulin-transferrin-selenium X (Invitrogen), and 10^-7 M dexamethasone (Sigma). After 4 h of inoculation, the cells were washed extensively with phosphate-buffered saline to remove hematopoietic cells and cell debris. Culture media were changed every 2 days. Hepatic cell lines, huH7, huH2.2, and HepG2, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

**Northern Blotting**—Total RNA from E12.5, E14.5, neonatal or adult liver, and cultured cells was extracted with Trizol solution (Invitrogen). 10-µg aliquots of total RNA were separated on a 1.2% agarose gel containing 2% formaldehyde and transferred onto a positively charged nylon membrane. After UV cross-linking, the membrane was hybridized with digoxigenin-labeling probes (cyclin D1, cyclin D2, cyclin D3, tyrosine aminotransferase (TAT), tryptophan oxygenase, or glyceraldehyde-3-phosphate dehydrogenase). The blot was treated with anti-digoxigenin antibody conjugated with alkaline phosphatase and then developed with CDP-Star (New England BioLabs).

**Vector Construction**—The EcoRI-XbaI cDNA fragments encoding G-CSFR-G133, G-CSFR-G133F2, G-CSFR-G133F3, and G-CSFR-G133F23 were inserted into the retroviral vector pMXII. The pMX-ires/GFP (pMI) retroviral vectors carrying cDNAs for ΔSTAT3, ΔSTAT5, RasV12, and RasN17 were constructed previously (34). The expression vector pME18S/STAT3-C was constructed by inserting the NotI-ApaI fragment of STAT3-C (a gift from Dr. J. F. Bromberg) (35). The integrity of these vectors was determined by sequencing and by digestion with restriction enzymes.

**Retroviral Infection**—The retroviral vectors were transduced into the retrovirus-packaging cell line (BOSC23 cells) by lipofection as described previously (29, 36). After 48 h of culture, the virus particles were enriched by centrifugation at 6,000 x g for 16 h at 4 °C. Pellets of the particles were resuspended in culture medium for fetal hepatocytes, and then the suspension was filtered through a 0.45-µm filter. Fetal hepatocytes were incubated with the viral solution for 3 days, and then the medium was changed to hepatocyte culture medium.

**Western Blotting**—Cultured hepatocytes were lysed in cell lysis buffer (25 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, leupeptin, Pefablock, 1% Triton X-100, and 0.5% Nonidet P-40). Ten micrograms of total cell lysate were used for SDS-PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with primary antibodies at room temperature for 2 h and washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20). They were then incubated with anti-mouse or rat IgG conjugated with horseradish peroxidase (Amersham Biosciences) at room temperature for 1 h, and the immune complex was visualized with the ECL system (Amersham Biosciences).

**Luciferase Assay**—Fetal hepatocytes were transduced with pME18S/STAT3-C and the reporter carrying two tandem repeats of APRE of α2-macroglobulin using LipofectAMINE plus (Invitrogen). As an internal control, the plasmid pRL containing the Renilla luciferase gene was co-transfected. To examine the effect of STAT3 on the cyclin D1 promoter in hepatic cells, fetal hepatocyte, huH7, HepG2, and huH2.2 were transiently transfected with pME18S/STAT3-C and the luciferase reporter linked to the human cyclin D1 promoter (−1745–Luc) (a gift from Dr. Y. Kanakura). Cells were cultured in medium for each cell type for 24 h and then lysed with passive lysis buffer (Promega). Luciferase activity was measured according to the technical manual for the Dual-Luciferase Reporter Assay System (Promega).

**Stable Transfectants of STAT3-C**—To establish STAT3-C stable transfectants of huH7, HepG2, and huH2.2, these cells were transfected with RcCMV-STAT3-C-neo (35), and stable transfectants of STAT3-C were selected by treatment of 800 µg/ml of G418 for 3 weeks. Since STAT3-C was tagged with FLAG, ectopic expression of STAT3-C was confirmed by Western blotting using anti-FLAG antibody (data not shown).

**Results**

**Developmental Regulation of the Expression of D-type Cyclins in the Liver**—As the expression of cyclin D1 is rapidly induced during liver injury, it is believed that D1 cyclin plays an essential role in the proliferation of hepatocytes during liver regeneration (32). However, neither the expression nor role of D-type cyclins in liver development had been clarified. We therefore first examined the expression of D-type cyclins during liver development by Northern analysis using RNA prepared from livers at different developmental stages (Fig. 1A). While both D1 and D3 cyclins were highly expressed in fetal liver until E14.5, their expression decreased along with the development of the liver. The expression level of cyclin D2 was rather low in the E12.5 and E14.5 livers and also further decreased during the development. In contrast, the expression of metabolic enzymes, TAT and tryptophan oxygenase, was induced in neonatal and adult liver, respectively (Fig. 1A). These results indicate that the expression of D-type cyclins is negatively regulated during liver development in vivo.

**OSM Down-regulates the Expression of Cyclin D1 and D2 in Fetal Hepatocytes in Vitro**—We previously demonstrated that OSM in combination with dexamethasone induces hepatic differentiation in vitro (20). Because OSM induces the expression of differentiation markers of neonatal liver such as TAT (20, 29), it is possible that OSM also regulates the expression of D-type cyclins in liver development. To test this possibility, we examined the mRNA expression of D-type cyclins in a primary culture of fetal hepatocytes at E14.5 (Fig. 1B). Consistent with...
Negative Regulation of Cyclin D Expression by OSM

OSM suggests that OSM down-regulates the expression of D1 and D2 cyclins by OSM. While cyclin D3 expression was down-regulated during liver development in vivo, the expression of D-type cyclins in the E14.5 liver in vitro (Fig. 1A), cyclin D1 was expressed abundantly in culture at day 0, and its expression was maintained in the absence of OSM for 7 days, while it was slightly decreased transiently at day 3 (Fig. 1B). In contrast, in the presence of OSM the expression of cyclin D1 was almost completely abrogated within 3 days. Although cyclin D2 was not expressed in culture at day 0, its expression increased gradually in the absence of OSM. OSM also suppressed the expression of cyclin D2, indicating the expression to be negatively regulated by the OSM signaling. While cyclin D3 expression was down-regulated during liver development in vivo, it was not affected by OSM in vitro, indicating that the OSM signaling is not sufficient to down-regulate cyclin D3 expression. We also investigated the mRNA expression of other cell cycle regulators including cyclin A, CDK 4, c-Myc, p21, and p27 in fetal hepatocytes; however, in no cases was it affected by OSM (data not shown). These results suggest that OSM down-regulates the expression of D1 and D2 cyclins in liver development.

The Docking Site for STAT3 in gp130 Is Required for the Negative Regulation of Cyclin D Expression by OSM—OSM functions through the OSM receptor, which consists of the OSM specific subunit (OSMR) and gp130, the common signal transducing subunit of the IL-6 family cytokines (22, 37, 38). As the OSM receptor, G133, lacks 144 amino acid residues from the C terminus of gp130, but still has both docking sites for SHP-2 and STAT3, stimulation with G-CSF leads to the activation of SHP-2/Ras and STAT3 signaling pathways in a manner similar to the full-length gp130 (Fig. 2A) (24, 25). As the G133F2 receptor lacks the docking site of SHP-2 due to substitution of the second tyrosine (Tyr) with phenylalanine (Phe), it activates only the STAT3 pathway. Conversely, G133F3, which lacks the docking site of STAT3, activates the SHP-2/Ras signaling alone (Fig. 2A). The G133F2/3 receptor that lacks both docking sites does not activate either signaling pathway. We constructed pMXII retroviral vectors carrying these chimeric receptors and expressed them in fetal hepatocytes as we previously reported. Fluorescence-activated cell sorter analysis using anti-G-CSFR antibody revealed that these receptors were expressed in 79–84% of fetal hepatocytes (Fig. 2B). Expression of the G133 receptor slightly reduced the expression of cyclin D1 and D2 in response to G-CSF as compared with the control level (Fig. 2C, lanes 3 and 4). Importantly, the G133F2 mutant receptor, which only activates the STAT3 signaling pathway, suppressed the expression of cyclin D1 and D2 in response to G-CSF to the same level as OSM (lanes 2 and 5). On the other hand, both G133F3 and G133F2/3, which do not activate STAT3 signaling, did not show such strong effect as G133F2 on cyclin expression (lanes 5, 6, and 7). These results suggest that the docking site of STAT3 in gp130 is essential for the down-regulation of cyclin D expression by OSM.

Dominant Negative STAT3 Relieves OSM-induced Suppression of Cyclin D Expression—To further confirm the role of STAT3 in the suppression of cyclin D expression, we next expressed STAT mutants using pMIG retroviral vectors. Ecotropic expression of the dominant negative form of STAT3 (STAT3ΔN terminus), which lacks the C-terminal transactivation domain, sustained the expression of D1 and D2 cyclins when OSM was present, indicating that ΔSTAT3 inhibits the negative effect of OSM on the expression of D1 and D2 cyclins (Fig. 3A, lane 3). In contrast, ΔSTAT5, which also lacks the C-terminal transactivation domain, had no inhibitory effect on their expression (Fig. 3A, lane 4). Although OSM also activates the SHP-2/Ras pathway, RasN17, a dominant negative form of Ras, did not affect the negative effect of OSM on the cyclin expression. Interestingly, however, activation of the Ras pathway by expression of RasV12, a constitutively active form, augmented the expression of cyclin D1 (Fig. 3A, lane 5). These results suggest that oncogenic Ras contributes to the induction of cyclin D1 expression, as previously shown by Wulf et al. (9). These results clearly demonstrate that the activation of STAT3 is indispensable for the suppression of the expression of D1 and D2 cyclins by OSM.

Activation of STAT3 Is Sufficient for the Suppression of Cyclin D Expression—The results described above clearly indicate that the STAT3 signaling pathway is necessary for the suppression of cyclin D expression during hepatic differentiation induced by OSM. To test whether the activation of STAT3 is sufficient for the down-regulation, we utilized transgenic mice that express STAT3ER, a fusion protein composed of the entire STAT3 with the modified ligand binding domain (G525R) of the estrogen receptor (Fig. 4A) (42). STAT3ER dimerizes in response to the synthetic estrogen receptor ligand, 4-hydroxytamoxifen (4HT), leading to the activation of the STAT3 signaling
pathway (42). Recently, Matsuda et al. generated a chimeric mouse with embryonic stem cells expressing STAT3ER, which had been maintained in the presence of 4HT instead of leukemia inhibitory factor (43). As the STAT3ER allele was transmitted to the germ line in this mouse, the offspring of the chimeric mouse expressed STAT3ER ubiquitously in almost all organs including the liver (data not shown). As E14.5 fetal hepatocytes derived from the chimeric mouse expressed STAT3ER proteins, we cultured the cells carrying the STAT3ER allele and investigated the expression of D-type cyclins (Fig. 4, A and B). Stimulation of the hepatocytes carrying the STAT3ER allele with 4HT suppressed the expression of D1 and D2 cyclins to the same level as did OSM (Fig. 4B, lanes 2–8). In contrast, 4HT did not down-regulate the cyclin expression in wild-type hepatocytes (lane 6), indicating that 4HT affects only the cells carrying the STAT3ER allele. Therefore, we conclude that the activation of STAT3 is necessary and sufficient for down-regulation of cyclin D1 and D2 expression in fetal hepatocytes.

**Role of STAT3 in Cyclin D1 Transcription**—Our results clearly establish that STAT3 acts as a negative regulator of cyclin D1 expression in fetal hepatocytes. However, it was recently demonstrated that STAT3 up-regulates the expression of cyclin D1 in rat 3Y1-immortalized fibroblasts and that

Fig. 2. The docking site for STAT3 in gp130 is essential for the suppression of cyclin D expression. A, structure of G-CSFR-gp130 chimeric receptors. The binding and activation of SHP-2 and STAT3 pathways in each receptor are summarized at the right. B, fluorescence-activated cell sorter analysis of fetal hepatocytes. Fetal hepatocytes were infected on day 0 with the pMXII retroviral vector carrying no signaling molecule, G133, G133F2, G133F3, or G133F2/3, and cultured for 4 days. Cells were harvested and dispersed in a single cell suspension and stained with anti-G-CSFR antibody. The expression of G-CSFR was analyzed using FACSCalibur. C, after infection, cells were cultured for 6 days without OSM and G-CSF (lane 1), with OSM (lane 2), or OSM and G-CSF (lanes 3, 4, 5, 6, and 7). Total RNA (10 μg) extracted from each culture was analyzed by Northern blotting to evaluate the expression of D-type cyclins. Vector only (lanes 1–3), G133, G133F2, G133F3, or G133F2/3 (lanes 4, 5, 6, or 7, respectively).

Fig. 3. Dominant negative STAT3 relieves the suppression of cyclin D expression by OSM. A, effects of various mutants of signal molecules on cyclin D expression. Fetal hepatocytes were infected on day 0 with the pMIG retroviral vector carrying no signaling molecule, ΔSTAT3 (lane 3), ΔSTAT5 (lane 4), RasV12 (lane 5), or RasN17 (lane 6) and cultured for 6 days in the presence (lanes 2–8) or absence (lane 1) of OSM. mRNA expression of D-type cyclins was determined by Northern blotting. B, ectopic expression of signal molecules in fetal hepatocytes. Cell extracts from each culture were analyzed by Western blotting using specific antibodies. ΔSTAT3 and ΔSTAT5 are smaller than the endogenous STAT protein as indicated. The asterisk shows a nonspecific band.
Developmental Regulation of Cyclin D Expression by STAT3

STAT3-C, a constitutively active form of STAT3, activates transcription from the human cyclin D1 promoter (−1745 CD11LUC) in 293T cells (35). These observations suggest that the role of STAT3 in cyclin D1 expression is dependent on cell type. To test this possibility, we investigated the effects of STAT3 on transcription from the cyclin D1 promoter using the luciferase reporter carrying the human cyclin D1 promoter (−1745-Luc) (44). STAT3-C has substitutions of two cysteine residues in the SH2 domain of STAT3 as shown in Fig. 5A. STAT3-C dimerizes spontaneously by forming a disulfide bond, translocates to the nucleus, and activates the transcription of target genes constitutively (35). Co-transfection of STAT3-C with the reporter containing two tandem repeats of APRE of the α2-macroglobulin gene into fetal hepatocytes led to the activation of transcription through APRE without OSM (Fig. 5B), indicating that STAT3-C acts as a constitutively active form of STAT3 in fetal hepatocytes as well as 3Y1 and 293T cells. Importantly, co-transfection of STAT3-C with the −1745-Luc reporter into fetal hepatocytes suppressed the cyclin D1 promoter activity as compared with control levels (−60%) (Fig. 5C). These results indicate that the effect of STAT3 on cyclin D1 transcription differs between fetal hepatocytes and the immortalized cell lines.

Although adult hepatocytes are quiescent, the expression of cyclin D1 is induced during liver regeneration as well as in hepatoma cells. In addition, STAT3 is up-regulated during liver regeneration and in hepatic tumor cells. These observations suggest that STAT3 enhances transcription of cyclin D1 in hepatoma and during regeneration of the liver. To test this possibility, we utilized established hepatic cell lines originating from cells in distinct stages of differentiation and examined the effect of STAT3 on cyclin D1 transcription by luciferase assay. We found that expression of STAT3-C induces activation of the cyclin D1 promoter in hepatocellular carcinoma, huH2.2 cells, and hepatoblastoma, HepG2 cells, although the levels of induction were different (Fig. 5C). In contrast, in the hepatoma cell line, huH2.2 cells, which are known to retain features of hepatocytes (45, 46), STAT3-C failed to activate the cyclin D1 promoter but rather slightly repressed the transcriptional activity (Fig. 5C). Consistent with the results of luciferase assays, Northern blot analysis revealed that mRNA expression of cyclin D1 was increased by STAT3-C in huH7 and HepG2, whereas it was slightly decreased by STAT3-C in HuH2.2 (Fig. 5D). These results indicate that the role of STAT3 in cyclin D1 transcription can be altered depending on the developmental stage of hepatocytes.

**DISCUSSION**

In mice, fetal hepatocytes first appear at E8.5 and proceed through a series of maturation steps including autonomous proliferation, cellular enlargement, tight cell-cell contacts, growth arrest, and functional maturation. In this study, we showed that the expression of D-type cyclins is down-regulated as liver development progresses (Fig. 1A). In the absence of OSM, the expression of D1 cyclin was sustained and D2 expression was rather increased. However, OSM significantly decreased the expression of D1 and D2 cyclins. These results suggest that OSM is a negative regulator of the expression of D1 and D2 cyclins during liver development in vivo. Although expression of cyclin D3 was down-regulated during liver development in vivo, OSM failed to down-regulate the expression of D3 cyclin in fetal hepatocytes in vitro. As we described previously, OSM induces the expression of a number of genes and functions, which are expressed in neonatal liver (20). However, the result that OSM failed to down-regulate D3 cyclin suggests that D3 expression is regulated by signals other than those induced by OSM and also that down-regulation of D3 expression is not required for the expression of various genes and functions in neonatal liver.

In fetal hepatocytes, OSM activates two major signals through gp130: the STAT3 and the SHP2/Ras pathways (29). As ΔSTAT3 inhibits induction of the expression of TAT, G6Pase, and CPS-I and also accumulation of glycogen by OSM, STAT3 mediates the OSM signaling for functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro. First, the G133F2 mutant receptor, which only activates STAT3, completely suppressed the expression of D1 and D2 cyclins in response to G-CSF (Fig. 2). Second, expression of dominant negative STAT3, ΔSTAT3, inhibited OSM-induced down-regulation of cyclin D expression (Fig. 3). Third, activation of STAT3ER in fetal hepatocytes derived from transgenic mice carrying the STAT3ER allele resulted in down-regulation of functional maturation of hepatocytes (29). In this paper, we provide evidence that the activation of STAT3 down-regulates cyclin D expression in fetal hepatocytes in vitro.
induces functional maturation. These observations also suggest that STAT3 functions not only as a positive regulator but also as a negative regulator of gene expression in fetal hepatocytes. Consistent with this notion, transcriptome analyses using STAT1-null cells showed that there are several genes that are regulated by STAT1 either positively or negatively in response to interferon-$
abla$ (47, 48). Thus, although positive effects of STATs have been extensively described, STATs are also involved in the negative regulation of gene expression. The mechanism of such negative regulation will be an interesting subject to study.

Ras is another key molecule that mediates the signals activated by gp130 in various cell types including fetal hepatocytes. We recently described that Ras, in particular K-Ras, contributes to the tight cell-cell contacts between hepatocytes by enhancing the formation of E-cadherin-based adherens junctions (30) and negatively regulates the STAT3 signaling pathway to induce the expression of hepatic differentiation markers (29). The results in this paper indicate that the Ras pathway is not required for OSM-induced down-regulation of cyclin D expression because the G133F3 chimeric receptor, which only activates SHP-2/Ras, did not affect cyclin D expression and expression of a dominant negative form of Ras failed to interfere with the suppression of cyclin D expression by OSM (Figs. 2 and 3). However, oncogenic Ras (RasV12) strongly induced expression of cyclin D1 in fetal hepatocytes (Fig. 3), consistent with previous observations that cyclin D1 is induced during Ras-dependent transformation (9). Therefore, the balance between STAT3 and Ras may be important to the progression of hepatic development.

It was previously demonstrated that STAT3 induces the transcription of cyclin D1 in the rat fibroblast cell line 3Y1, correlating STAT3 activation with tumorigenesis (35). In contrast, we show here that STAT3 down-regulates cyclin D1 transcription during hepatic development. Because the DNA segment of the human cyclin D1 promoter we used for the transactivation assays was the same 1878-bp $Pvu II$ fragment used by Bromberg et al. (35), it is clear that the effect of STAT3 on cyclin D1 transcription differs between fetal hepatocytes and immortalized cells. Consistent with this notion, co-transfection of STAT3-C with the $1745$-Luc reporter into established hepatoma cell lines, huH7 and HepG2 (46, 49), enhanced the transcription of the cyclin D1 promoter. Interestingly, however, STAT3-C failed to enhance transcription from the D1 promoter in the huH2.2 cell line that retains characteristics of hepatocytes (Fig. 5C). Consistent with the idea that activation of STAT3 is correlated with tumorigenesis, it was recently demonstrated that SOCS-1, a negative regulator of the Jak/STAT pathway, is silenced by methylation in 65% of human primary hepatocellular carcinomas, leading to the constitutive activation of STAT3 and outgrowth of hepatocytes (50). Moreover, Cressman et al. reported that IL-6-deficient mice exhibit defects in STAT3 activation and in cyclin D1 induction after partial hepatectomy (28). Therefore, it is likely that STAT3 acts as a negative regulator of cyclin D1 transcription during fetal liver development, whereas it positively regulates cyclin D1 expression in hepatoma cells and at the initial phase of liver regeneration. These findings again suggest that the cyclin D1 gene is an important target of STAT3 in hepatocytes and that its regulation by STAT3 varies dependent on the cell stage, i.e. proliferation or differentiation. Perhaps an additional factor is...
involved in the cell type-specific response, and expression of such a factor may be developmentally regulated.

In this study, we showed that the expression of cyclin D is down-regulated along with liver development and that STAT3 mediates the OSM signaling for the down-regulation of D1 cyclin during the differentiation. Furthermore, STAT3 differentially regulates transcription of D1 cyclin in hepatocytes depending on the developmental stage. The mechanism underlying such differential regulation will be interesting and important to the study of liver development.

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