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Pituitary Transcription Factor-1 Induces Transient Differentiation of Adult Hepatic Stem Cells into Prolactin-Producing Cells *in Vivo*

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A subset of transcription factors function as pivotal regulators of cell differentiation pathways. Pituitary transcription factor-1 (Pit-1) is a tissue-specific homeodomain protein that specifies the development of pituitary somatotropes and lactotropes. In this study, adenovirus was used to deliver rat Pit-1 to mouse liver. Pit-1 expression was detected in the majority (50–80%) of hepatocyte nuclei after tail vein injection (2×10^9 plaque forming units). Pit-1 activated hepatic expression of the endogenous prolactin (PRL), GH, and TSHβ genes along with several other markers of lactotrope progenitor cells. Focal clusters (0.2–0.5% of liver cells per tissue section) of periportal cells were positive for PRL by immunofluorescent staining. The PRL-producing cells also expressed proliferating cell nuclear antigen as well as the hepatic stem cell markers (c-Kit, Thy1, and cytokeratin 14). These data indicate that Pit-1 induces the transient differentiation of hepatic progenitor cells into PRL-producing cells, providing additional evidence that transcription factors can specify the differentiation pathway of adult stem cells. (*Molecular Endocrinology* 19: 964–971, 2005)

THE ANTERIOR PITUITARY gland develops from the somatic ectoderm in the roof of the oral cavity known as Rathke's pouch and consists of six hormone-secreting cell types. The pituitary cell types have been classified into three major cell lineages [GH-prolactin (PRL)-TSH, proopiomelanocortin, and FSH/LH] controlled by specific combinations of transcription factors and extracellular growth factors (1). Pituitary transcription factor-1 (Pit-1) is required for terminal differentiation of the GH-PRL-TSH cell lineage (1–3). In addition to specifying this cell lineage, Pit-1 directly stimulates transcription of GH, PRL, and TSHβ genes, as well as several regulatory genes involved in cell growth and homeostatic control (4–6). Pit-1 mutations cause a dwarf phenotype lacking GH, PRL, and TSH in mice (7) and humans (8).

Adult stem cells are generally considered to be tissue-restricted in their developmental potential (9). However, there have been recent examples in which stem cells cross tissue-specific barriers. Neural stem cells have been shown to transdifferentiate into hematopoietic elements (10) or myotubes (11) after transplantation into animals. Recently, insulin-producing cells have been produced in mouse liver after adenoviral-mediated delivery of islet-specific transcription factors (12, 13). In this instance, liver parenchymal cells and pancreatic endocrine cells are of similar embryonic origin, perhaps facilitating the transdifferentiation process. Consistent with this idea, the induction of diabetes by streptozotocin destruction of the pancreatic β-cells is associated with the induction of a small number of insulin-producing cells in the liver (13).

In this study, we used the Pit-1 to test whether it is feasible to induce hepatic progenitors to differentiate into other endocrine cell types. After adenoviral delivery of Pit-1, the endogenous genes for PRL, GH, and TSHβ were activated in the liver, indicating that Pit-1 can induce differentiation in a tissue that does not normally express these hormones.

RESULTS

Pit-1 Induces Activation of Pituitary Hormone Genes in the Liver

Pit-1 gene was expressed in 50–80% of hepatocytes 3 to 7 d after Ad-Pit-1 injection [2×10^9 plaque-forming units (PFU)] into the mouse tail vein. Pit-1 expression was greatest in the hepatic lobules, but scattered expression was also observed in bile ductal cells and small cells in the periportal area (Fig. 1A). RT-PCR analysis of total RNA using rat-specific primers confirmed rat Pit-1 (rPit-1) expression in livers of mice injected with Ad-Pit-1. In female mice, Pit-1 activated expression of endogenous genes for a subset of mouse pituitary hormones (Fig. 1C). All of the Pit-1-positive livers expressed mouse PRL mRNA. Mouse GH and TSHβ mRNAs were detected in 36% (5 of 14) and 50% (7 of 14) of injections,

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Abbreviations: D2R, Dopamine 2 receptor; FGF, fibroblast growth factor; PCNA, proliferating cell nuclear antigen; PFU, plaque-forming unit(s); Pit-1, pituitary transcription factor-1; PRL, prolactin; rPit-1, rat Pit-1; TRH-R, TRH receptor.

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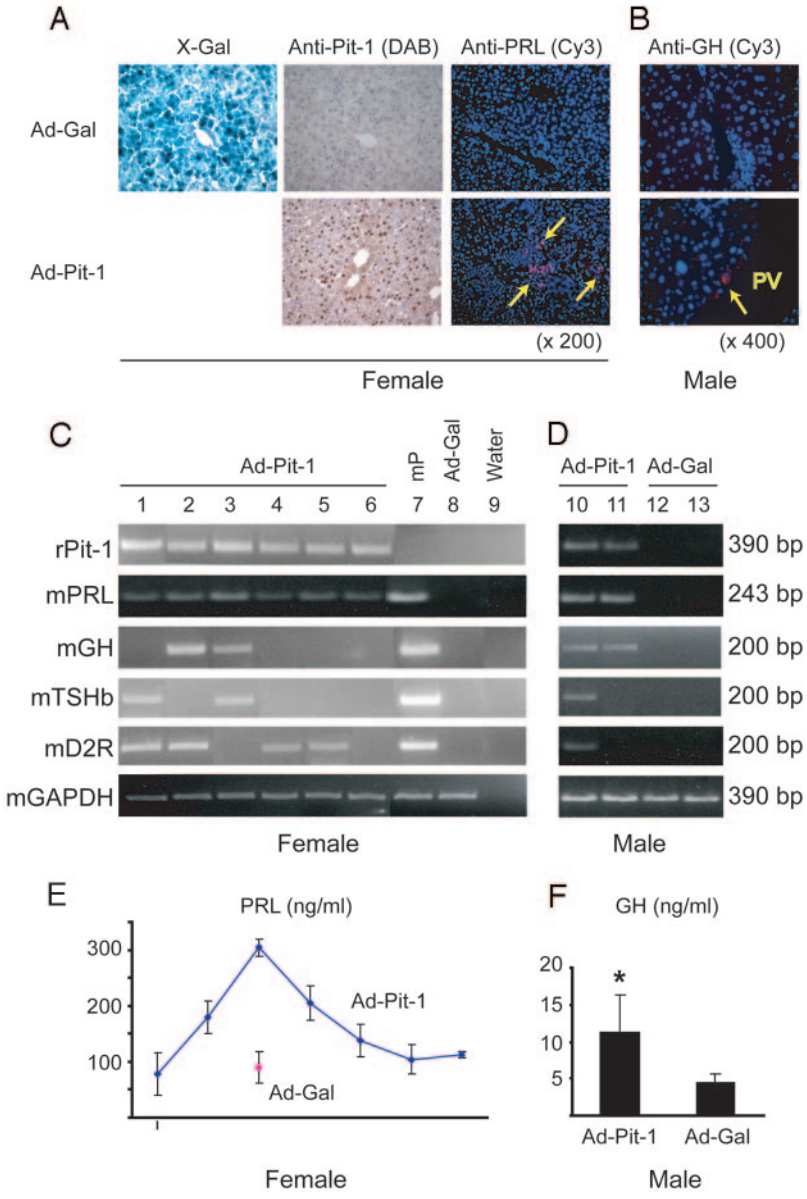

Fig. 1. Activation of Pituitary Hormone Genes in the Livers of Mice Treated with Ad-Gal or Ad-Pit-1

Mice were killed 7 d after tail vein injection of 2×10^9 PFU of recombinant adenoviruses, and liver was removed for RNA isolation and histological preparation. A and B, Histological analysis of female (A) and male (B) mouse livers after treatment of adenoviral vectors. Removed liver of mice injected with Ad-Gal was embedded in tissue freezing medium, and frozen section was prepared. Slide was incubated with X-Gal substrate solution to visualize expression of the β-galactosidase gene (*top left panel*). Sections of paraffin-embedded tissues 4 μm in thickness were used in immunohistochemical staining for Pit-1 and immunofluorescent staining for PRL and GH. All of the photomicrographs are $\times$200 magnification, except GH ($\times$400). C and D, RT-PCR analysis to determine the expression of transduced rPit-1 gene and the activation of mouse endogenous pituitary genes in female (C) and male (D) mice. An aliquot of each PCR was resolved by electrophoresis on 1.75–2.0% agarose gels, and DNA products were visualized with ethidium bromide. Lanes 1–6, 10, and 11, Ad-Pit-1 injection; lane 7, normal mouse pituitary; lanes 8, 12, and 13, Ad-Gal injection; lane 9, no DNA control. E and F, Serum levels for mouse PRL (E) and GH (F). Serum PRL and GH were measured by RIA using mouse-specific antibodies. Each time point was the mean (n =6) $\pm$ SD. *, $P < 0.05$.

respectively. Dopamine 2 receptor (D2R) mRNA, which is specific to lactotropes, was expressed in 57% (8 of 14) of injections. TRH-receptor (TRH-R) mRNA expression was detected in 7.1% (1 of 14). In male mice (Fig. 1D), GH and PRL mRNAs were detected in all of the Pit-1-positive livers. TSHβ and D2R mRNA were detected in 50% (3 of 6) of injections. Other pituitary hormones (ACTH, LH, and FSH), which are not characteristic of the Pit-1 cell lineage, were not detected in either female or male mice (data not shown). There was no hepatic expression of mouse pituitary-specific genes in uninjected mice or control Ad-Gal-injected mice.

Immunofluorescent staining for mouse GH, PRL, and TSHβ was performed to investigate whether the expressed mRNAs were effectively translated into hormones in the liver cells. Focal cell clusters (0.2–0.5% of the hepatic cells in the section) positive for PRL were restricted to the periportal areas in female liver (Fig. 1A). Serum PRL was measured by RIA using a mouse-specific antibody. Serum PRL reached a peak at d 7 (304.0 $\pm$ 15.3 ng/ml) and then decreased to normal by d 28. Serum PRL was not increased in Ad-Gal-treated mice (89.2 $\pm$ 28.3 ng/ml at d 7) (Fig. 1E). In contrast to PRL, GH and TSHβ immunopositive cells were not detected in female livers that expressed these mRNAs (data not shown), suggesting that GH and TSH proteins are absent or very low in these cells. Consistent with these findings, serum GH and TSH levels were not increased in the Ad-Pit-1-injected mice (data not shown). In male mice, GH-positive cell clusters were detected in the portal area (Fig. 1B), but PRL or TSHβ immunopositive cells were absent. Serum GH levels (11.3 $\pm$ 4.4 ng/ml) were greater in Ad-Pit-1-injected male mice than in Ad-Gal-injected mice (5.1 $\pm$ 1.2 ng/ml) (Fig. 1F). However, other pituitary hormone levels were not elevated.

PRL levels in serum might also reflect Ad-Pit-1 expression in nonhepatic tissues. We previously observed that systemic administration of adenoviral vector results in transgene expression mainly in liver and lung, with low levels of expression in the glomeruli of kidney, but not in the pituitary (14). Consistent with these results, rat Pit-1 expression was also detected in kidney by RT-PCR, and rare glomerular cells were immunopositive for Pit-1. However, no pituitary hormone expression was detected in the kidney (data not shown).

To further assess the possibility that Ad-Pit-1 might transactivate endogenous pituitary genes, mice were hypophysectomized before injection with a reduced amount of adenoviruses (1×10^9 PFU) because of vulnerability to stress. After injection of Ad-Pit-1, Pit-1 was expressed in 30–50% of liver cells (Fig. 2A). RT-PCR confirmed expression of mRNAs for PRL, GH, TSHβ, and D2R (Fig. 2B), and immunopositive PRL cells were found near the portal area in the liver (Fig. 2A). Serum PRL was elevated (98.6 ng/ml) compared with Ad-Gal-injected mice ($<$0.1 ng/ml). GH and TSH were not elevated in either group of animals (data not shown). Thus, the increased serum PRL level after Ad-Pit-1 injection is independent of Pit-1- or stress-induced PRL production by preexisting pituitary lactotropes.

Differentiation of Hepatic Stem Cells into PRL-Producing Cells

PRL-positive cells were oval-shaped and smaller than normal hepatocytes 5 to 7 d after Ad-Pit-1 treatment. These cells had a high nuclear/cytoplasmic ratio, suggesting that they were proliferating. Immunofluorescence for proliferating cell nuclear antigen (PCNA) demonstrated that the PRL-positive cells were also positive for PCNA (Fig. 3), consistent with a proliferating cell population. Of note, PCNA was detected throughout the livers of mice injected with either Ad-Gal or Ad-Pit-1 (Fig. 3), presumably because the adenoviral injection induced hepatitis and hepatic regeneration. PCNA was seen in both normal hepatocytes and in the small cell populations (data not shown), indicating that regeneration occurs in both normal hepatocytes and hepatic progenitor populations. Although regeneration activity occurred in normal hepatocytes transduced with rPit-1, PRL positivity was not detected in these cells, indicating that the potential for PRL expression is possessed by a limited subpopulation of liver cells. The origin of these PRL-positive cells was investigated using double-labeled immunofluo-

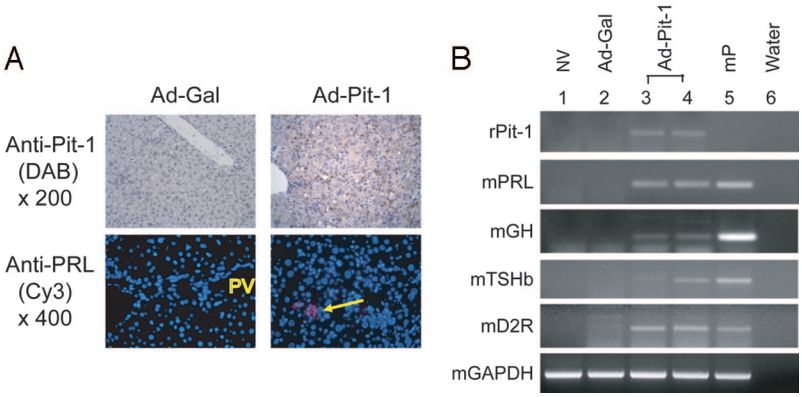

Fig. 2. Activation of Pituitary Hormone Genes in the Livers of Hypophysectomized Female Mice Treated with Ad-Pit-1

Mice were killed 7 d after tail vein injection of 1×10^9 PFU of recombinant adenoviruses, and liver was removed for RNA isolation and histological preparation. A, Paraffin sections (4 μm) were used for immunohistochemical and immunofluorescent staining to detect Pit-1 (*upper panel*, $\times$200) and PRL (*lower panel*, $\times$400), respectively. B, RT-PCR analysis was performed to determine the expression of transduced rPit-1 gene and the activation of mouse endogenous pituitary genes. Lane 1, Uninjected control, no virus (NV); lane 2, Ad-Gal injection; lanes 3 and 4, Ad-Pit-1 injection; lane 5, normal mouse pituitary (mP); lane 6, no DNA control.

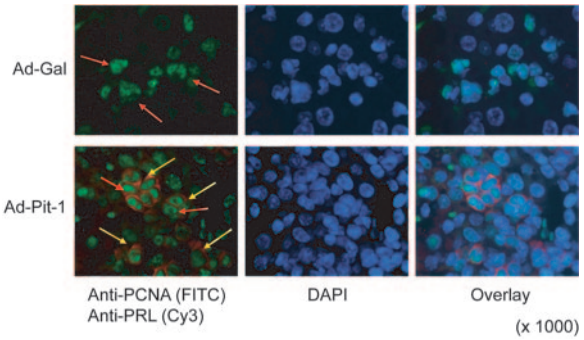

Fig. 3. Colocalization of PCNA and PRL in Livers of Mice Treated with Ad-Gal or Ad-Pit-1

Paraffin sections of livers of mice at d 7 after treatment were analyzed by double immunofluorescence staining for PRL and PCNA. Cells with green fluorescent nuclei express PCNA (*red arrows*), and cells with *red* fluorescent cytoplasm express PRL (*yellow arrows*). All of the photomicrographs are high magnification views (×1000).

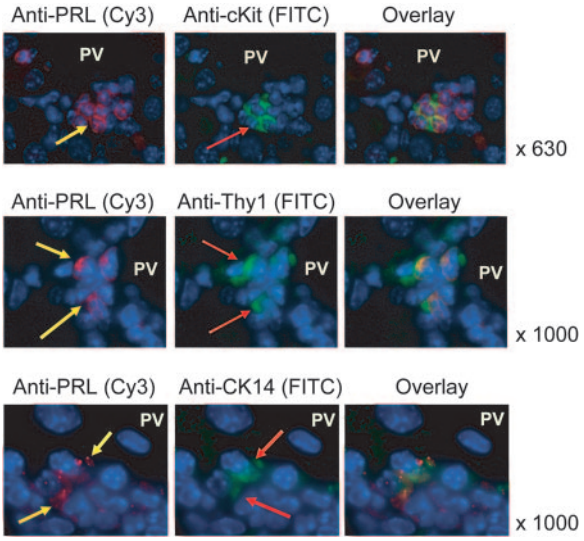

Fig. 4. Colocalization of PRL with Stem Cell Markers in Livers of Mice Treated with Ad-Pit-1

Paraffin sections of livers of mice at d 5 after treatment were analyzed by double immunofluorescence staining for PRL with c-Kit, Thy-1, or cytokeratin 14. Cells with *red* fluorescent cytoplasm express PRL (*yellow arrows in left panels*), and cells with *green* fluorescent cytoplasm (*red arrows in center panels*) express c-Kit (*top*), Thy-1 (*middle*), or cytokeratin 14 (*bottom*). PV, Portal vein. Magnification, *top panels*, ×630; *middle and bottom panels*, ×1000.

rescence staining. At the earlier stages (d 3–5), PRL-positive cells were also positive for the hepatic stem cell markers c-Kit, Thy-1, and cytokeratin 14 (Fig. 4). However, these markers were no longer present in PRL-positive cell clusters after d 7, suggesting that the PRL-positive cells are derived from hepatic stem cells.

Double-labeled immunofluorescence for PRL and Pit-1 was performed to investigate the relationship between Pit-1 and PRL-positive cells. At d 3 after Ad-Pit-1 injection, Pit-1 was expressed in the nucleus of single or double cells that were also positive for PRL (Fig. 5). However, by d 7, there was an unexpected dissociation of Pit-1 and PRL expression. Pit-1 continued to be expressed in isolated normal hepatocytes, but most PRL expression was seen in small cell clusters (32–64 cells) that did not express Pit-1 (Fig. 5), suggesting episomal dilution of adenovirus-mediated Pit-1 gene transduction.

Time-Course of PRL Expression

PRL expression in mouse livers was analyzed at d 3, 5, 7, 14, 21, 28, and 56 to investigate the fate of PRL-positive cells over time. By RT-PCR, the adenoviral-delivered rPit-1 gene expression diminished by d 21 and its expression was undetectable by d 28. The activated expression of endogenous pituitary-specific genes (mPRL, mGH, mTSHβ, and mD2R) decreased in parallel with the decline of Pit-1 gene expression (Fig. 6A).

Immunohistochemistry showed that Pit-1 expression peaked at d 3–7, diminished at d 14, and disappeared by d 21 (Fig. 6B). Single or double cells were positive for PRL in the periportal area at d 3. PRL-positive cells formed a cluster of small cells with a high nuclear/cytoplasmic ratio at d 5–7. At d 14, PRL positivity spread out from the hepatic lobule, and it diminished by d 21. Pit-1 and PRL were no longer detected at d 28 and 56 (data not shown).

DISCUSSION

In this study, we have shown that adenoviral gene delivery of the Pit-1 induces the differentiation of mouse liver cells into pituitary progenitors that express an array of genes characteristic of the early lactotrope lineage (PRL, GH, TSHβ, D2R, TRH-R). Pituitary genes

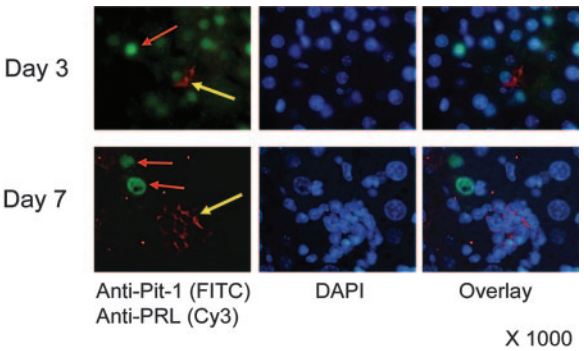

Fig. 5. Colocalization of PRL and Pit-1 in Livers of Mice Treated with Ad-Pit-1

Paraffin sections of livers of mice at d 3 and 7 after treatment with Ad-Pit-1 were analyzed by double immunofluorescence staining for PRL and Pit-1. Cells with *green* fluorescent nuclei express Pit-1 (*red arrows in left panels*), and cells with *red* fluorescent cytoplasm express PRL (*yellow arrows in left panels*). All of the photomicrographs are high magnification views (×1000).

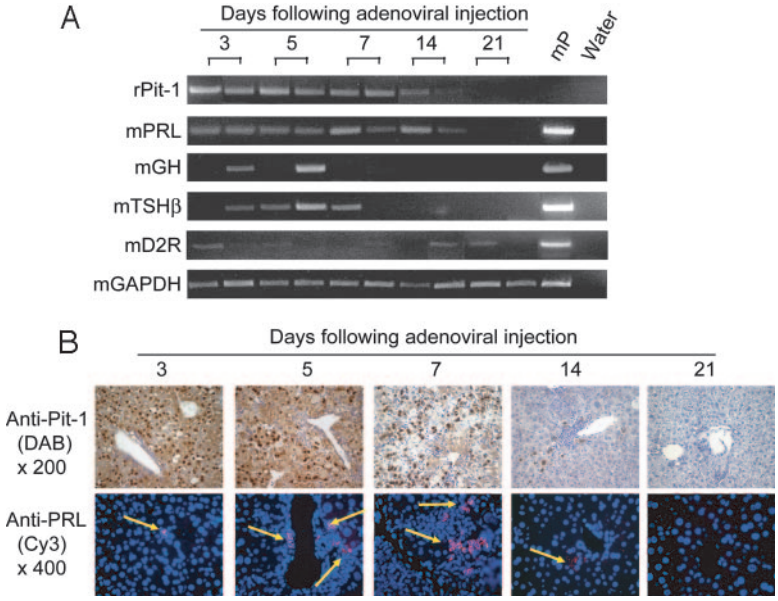

Fig. 6. Time Course of PRL Cells in Livers of Mice Treated with Ad-Pit-1

After injection of recombinant adenoviruses, mice were killed, and livers were removed at different time points (d 3, 5, 7, 14, and 21). A, Total RNA extracted from livers was subjected to RT-PCR analysis to determine the expression of transduced rPit-1 gene and the activation of mouse endogenous pituitary genes. mP, Normal mouse pituitary; Water, no DNA control. B, Histological analysis of liver. Sections (4 μm) of paraffin-embedded tissues were used in immunohistochemical and immunofluorescent staining for Pit-1. Magnification, *upper panels*, $\times$200; *lower panels*, PRL expression, $\times$400.

from other cell lineages, such as corticotropes (ACTH) or gonadotropes (LH, FSH), were not induced, indicating lineage-specific effects of Pit-1. Although Pit-1 expression was detected in 50–80% of hepatocytes, PRL-positive cells comprised only 0.2–0.5% of the liver cells. This selected cell population was positive for hepatic stem cell markers (Thy-1, c-Kit, and cytokeratin 14), suggesting that the potential for differentiation is conferred by a progenitor cell population in the liver.

It is notable that Pit-1 expression is transient and is absent from many of the cells that express PRL. The transient expression of Pit-1 is consistent with the properties of adenovirus-delivered genes, which are episomal and become degraded after several weeks. As the progenitor cells divide, Pit-1 may be progressively diluted, until it is finally lost entirely. The transient exposure to Pit-1 appears to be sufficient to direct cells into the lactotrope lineage. However, this population is not sustained long term and ultimately disappears after about 14–21 d.

The hepatic progenitor cells include the so-called oval cells located in the canal of Hering (15). In some types of liver damage, these small cells, which have a high nuclear/cytoplasmic ratio, emerge in the portal zone, proliferate extensively, migrate into the lobule, and eventually become differentiated hepatocytes (15). PRL-positive cells appear to follow a similar course. After injection of Ad-Pit-1, small PRL-positive cells appeared in the periportal area at d 3, began to proliferate, and formed clusters of small cells by d 5–7. By d 14, large PRL-positive cells spread toward the central vein.

The hepatic oval cells are known to be bipotential and retain the ability to differentiate into both the bile duct epithelial and hepatocyte lineages *in vitro* (16). They express markers characteristic of both bile duct epithelium (cytokeratin 19) (17) and hepatocytes (albumin). Oval cells are also immunoreactive to antibodies generally associated with hematopoietic lineages including Thy-1, CD34, and c-Kit, suggesting that oval cells originate from hematopoietic stem cells (18). The PRL-positive cells were immunopositive for Thy-1 and c-Kit, suggesting that the oval cells are the likely progenitor population for the PRL-producing cells.

Although stem cell plasticity has been demonstrated in many tissues *in vivo* (10, 11, 19–21), it is likely that multiple factors are required to induce cell-specific differentiation. Hepatic oval cells can be differentiated to insulin-producing pancreatic islet cells by culturing in high-glucose medium (22), presumably because glucose provides a stimulus for insulin production. Primary neural stem cells, when cocultured with differentiating myotubes, differentiate into myocytes (23). Hepatic oval cells transdifferentiate into microglial cells after transplantation to the neonatal mouse brain (24). Kojima *et al.* (13) demonstrated that coinjection of adenoviral vectors carrying Neuro-D or betacellulin facilitated more efficient differentiation of liver cells into insulin-producing cells than either factor alone. These results indicate that the microenvironment and cell extrinsic factors are likely to play a role in stem cell

plasticity, although the transduced transcription factor is a key regulator of cell fate.

We hypothesize that hepatic stem cells transduced with the Pit-1 gene might be stimulated by local factors to differentiate further into pituitary lineage cells. Maturation of the Pit-1-directed cell lineage into somatotropes, lactotropes, or thyrotropes requires the additional expression of GHRH receptor, which stimulates GH production (25); estrogen receptor, which stimulates PRL production (26); and thyrotrope embryonic factor, which stimulates TSHβ production (27). Liver parenchymal cells are known to express estrogen receptor but not GHRH receptor or thyrotrope embryonic factor (28), perhaps explaining the preferential production of PRL *vs.* GH or TSH in female mice. Interestingly, GH production was seen in male mice transduced with Pit-1. It has been suggested that cell type-specific expression of GH and PRL is determined by Pit-1 association with either coactivators or corepressors, directly or indirectly (1). The preferential production of GH in the male and PRL in the female liver might be related to sex steroid effects, but this remains to be tested directly. The hepatitis associated with adenovirus infection leads to hepatic regeneration, which induces a variety of growth factors such as TGFβ3 and fibroblast growth factor (FGF)-2 (29–31). FGF2 is known to stimulate PRL promoter activity and PRL secretion in normal and tumorous lactotrope-origin GH4 cells (32, 33). FGF-2 has also been shown to initiate lactotrope differentiation and maintain PRL expression in mouse pituitary GHFT cells that harbor Pit-1 (34). The ultimate decline of PRL expression after about 21 d demonstrates that adenoviral-delivered Pit-1 is not sufficient to sustain long-term lactotrope cell regeneration. This likely reflects the transient nature of Pit-1 expression, the absence of induction of endogenous mouse Pit-1, and loss of paracrine stimulation of growth factors (data not shown). In contrast, adenoviral-delivered rat pancreatic-duodenal homeobox-1 induces expression of mouse pancreatic-duodenal homeobox-1, resulting in long-term production of insulin-producing cells in the liver (35). Viruses designed to integrate Pit-1 into the genome may be capable of inducing PRL-producing cells for longer periods of time.

In summary, we have shown that adenoviral gene delivery of Pit-1 transcription factor induces transient differentiation of mouse liver cells into pituitary PRL-producing cells. The PRL-producing cells are most likely differentiated from hepatic stem cells in a process driven by the Pit-1 transcription factor, perhaps in conjunction with the expression of local regenerative growth factors (TGF-β3 and FGF-2). These data further elucidate pathways of stem cell plasticity and suggest strategies for using developmental transcription factors to induce adult stem cells to differentiate along specified lineage pathways.

MATERIALS AND METHODS

Generation of Adenoviral Vectors

A cassette containing the rPit-1 cDNA (cloned by RT-PCR using total RNA extracted from GH3 cells) driven by the cytomegalovirus promoter/enhancer with a simian virus 40 polyadenylation sequence was subcloned into an adenoviral transfer plasmid (36) based on pCDNA3 (Invitrogen, Carlsbad, CA). This plasmid was used to generate recombinant adenovirus, Ad-Pit-1, as described previously (36). Ad-Gal (14), which contains β-galactosidase driven by cytomegalovirus promoter, was used as a control.

Injection of Adenovirus *in Vivo*

Six- to 7-wk-old mice (C57BL6, 25–28 g in body weight) were housed in a barrier facility with a 12-h light, 12-h dark cycle and were fed a standard unrestricted diet. Female mice were injected with 2×10^9 PFU of recombinant adenoviruses through the tail vein and were euthanized at various time points (d 3, 5, 7, 14, 21, 28, and 56). Male mice were injected at 8 wk of age and were killed at d 7. Hypophysectomized female mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a barrier facility for 7 d with hydrocortisone (Sigma, St. Louis, MO) replacement (500 μg/kg body wt sc daily) before injection with Ad-Pit-1 or Ad-Gal (1×10^9 PFU). After euthanization, the pituitary region was examined to confirm complete hypophysectomy. Blood was drawn from the retroorbital sinus for measurements of mouse pituitary hormones. For the isolation of hepatic RNA, the liver was immediately frozen in liquid nitrogen and stored at −80 C. Remaining liver samples were fixed in neutral buffered formalin and embedded in paraffin for immunohistochemical staining. Serum levels for mouse PRL, GH, and TSHβ were measured by RIA in hormone assay core at the Vanderbilt Mouse Metabolic Phenotyping Center (Nashville, TN) using mouse-specific antibodies. All studies using mice were approved by the Northwestern University Medical School Animal Care and Use Committee.

RT-PCR Measurement of Gene Expression

Total RNA was isolated from frozen liver tissues using TRIZOL reagent (Invitrogen) as described by the manufacturer. A total of 20 μg of RNA was treated with DNAse-I (Promega, Madison, WI) for 30 min at room temperature. Random hexamers and avian myeloblastosis virus reverse transcriptase (RT) were used to synthesize cDNA using 10 μg of DNAse-I-treated RNA. A portion (1/40) of the cDNA solution was used for amplification of rPit-1, mPRL, mGH, mTSHβ, mTRH-R, mD2R, and mGAPDH. Cycle conditions were: 2 min hot start at 96 C, followed by 35 cycles of 1 min at 94 C, 1 min at 55 C, followed by 1.5 min at 72 C, and a final extension at 72 C for 10 min. An aliquot (20%) of each PCR was resolved by electrophoresis on 1.75–2.0% agarose gels, and DNA products were visualized with ethidium bromide. Oligonucleotides used for PCR amplification were as follows: rPit-1 (385 bp), forward 5′-GCGGAGGACCCAACAGCCTCTGAATT-3′, and reverse 5′-CCTTAGCGGCGATACTGATAGTTGTC-3′; mPRL (243bp), forward 5′-ATCAATGACTGCCCCACTT-3′, and reverse 5′-GCTAATTATCTTCTCAACTC-3′; mGH (200 bp), forward 5′-ATTTTCACCAACAGCCTGATG-3′, and reverse 5′-GCAGCCCATAGTTTTTGAGC-3′; mTSHβ (200 bp), forward 5′-CAAGCAGCATCCTTTTGTATTC-3′, and reverse 5′-CCACCGTTCTGTAGATGAAGTC-3′; mD2R (180 bp), forward 5′-GTCCTTCACCATCTCTTGCC-3′, and reverse 5′-CTGCTACGCTTGGTGTTGAC-3′; mTRH-R (200 bp), forward 5′-CCAGGAAAATTGGTTCTTGC-3′ and reverse 5′-TGCTGAAGCGGTCTGACTC-3′; and mGAPDH (390 bp), forward 5′-CCAAAGTTGTCATGGATGA-

C-3′ and reverse 5′-CCCTTCATTGACCTCAACTA-3′. The primers designed to detect the ectopic rat Pit-1 expression do not amplify the mouse homolog.

Immunohistochemistry and immunofluorescence

Sections of paraffin-embedded tissues 4 μm in thickness were used for immunostaining. Immunohistochemistry using an ABC kit and DAB chromogen (Vector Laboratories, Inc., Burlingame, CA) was performed to detect rPit-1. The expression of PRL, GH, and TSHβ was examined using immunofluorescence staining. Double immunofluorescence was performed to detect the colocalization of PRL with Pit-1, PCNA, c-Kit, Thy1, or cytokeratin 14. The primary antibodies, including rabbit polyclonal anti-Pit-1 (sc-442, 7.5μg/ml), goat polyclonal anti-PRL (sc-7807, 7.5 μg/ml), goat polyclonal anti-GH (sc-10364, 7.5 μg/ml), goat polyclonal anti-TSHβ (sc-7815, 7.5μg/ml), mouse monoclonal anti-PCNA (sc-56, 5.0 μg/ml), rat monoclonal anti-c-Kit (sc-19619, 7.5 μg/ml), and rabbit polyclonal anti-Thy1 (sc-9163, 7.5 μg/ml), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other primary antibody included mouse monoclonal anticytokeratin 14 (RTU-LL002, NovoCastra Laboratories Ltd., Newcastle, UK). Secondary staining reagents including Cy3-conjugated antigoat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), biotinylated secondary antibodies (antirabbit, -goat, -mouse, or -rat IgG, Vector Laboratories), and fluorescein-streptavidin (Vector Laboratories) were titrated individually for minimal nonspecific signal. For the detection of Pit-1, PCNA, and cytokeratin 14, slides were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Trypsin (T-7168, Sigma) treatment was performed to detect c-Kit and Thy-1. The MOM kit (Vector Laboratories) was used to minimize nonspecific signal from the mouse-origin primary antibodies on mouse tissue. To minimize the autofluorescence, slides were incubated in 70% ethanol supplemented with 0.1% sudan black B for 15 min and washed vigorously in running water. Fluorescent mounting media (Vector Laboratories) containing 4′,6′ diamidino-2 phenylindol was used for nuclei-counterstaining and mounting. Images were obtained using a Zeiss Axioskop fluorescent microscope (Carl Zeiss Microimaging Inc., Thornwood, NY).

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