Epiregulin promotes the emergence and proliferation of adult liver progenitor cells

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CURRENTLY, LIVER TRANSPLANTATION is the only therapeutic option for rescue of patients with end-stage liver disease. However, the shortage of organ donors has limited its application for most patients, including those in Japan, suggesting the need for an alternative therapeutic option. One such promising option for liver regeneration is cellular transplantation, using resources such as pluripotent stem cells, embryonic stem cells, umbilical cord blood cells, bone marrow cells (BMCs), liver cells, and liver progenitor cells (LPCs). We have been focusing on BMCs and LPCs, the latter pattern of regeneration involving LPCs is more important in patients with advanced liver failure. However, the precise relationship between epiregulin and its effects on LPCs has remained unclear.

The aim of the present study was to clarify the role of epiregulin during liver regeneration using a mouse model. Our results suggested that epiregulin plays a role in liver regeneration by promoting cellular proliferation and the emergence of LPCs.
**Table 1. Patients’ characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Acute Liver Failure, n = 24</th>
<th>Acute Hepatitis, n = 24</th>
<th>Liver Cirrhosis, n = 24</th>
<th>Chronic Hepatitis, n = 24</th>
<th>Control, n = 24</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>47 ± 14.6</td>
<td>48.5 ± 12.2</td>
<td>48.1 ± 11.1</td>
<td>48.6 ± 12.9</td>
<td>48.8 ± 15.7</td>
<td>0.99</td>
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<tr>
<td>Sex, m/f</td>
<td>10/4</td>
<td>14/10</td>
<td>13/11</td>
<td>14/10</td>
<td>14/10</td>
<td>0.88</td>
</tr>
<tr>
<td>Total bilirubin, mg/dl</td>
<td>9.8 [2.4–32.4]</td>
<td>3.8 [0.4–40]</td>
<td>1.7 [0.4–32]</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.4</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>ALT, IU/l</td>
<td>1377 [37–5690]</td>
<td>545 [56–4640]</td>
<td>31 [8–122]</td>
<td>38 [13–129]</td>
<td>17.7 ± 6.5</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Plt, 1.0 × 10^11/μl</td>
<td>11 ± 6.8</td>
<td>23.3 ± 10.2</td>
<td>11.6 ± 7.5</td>
<td>18.2 ± 5.1</td>
<td>25.6 ± 8.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>PT, %</td>
<td>33.7 ± 10.6</td>
<td>92.8 ± 28.2</td>
<td>68.8 ± 18.8</td>
<td>107.2 ± 8.9</td>
<td>—</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>PT-INR</td>
<td>1.9 ± 0.3</td>
<td>1.08 ± 0.18</td>
<td>1.27 ± 0.24</td>
<td>0.65 ± 0.05</td>
<td>—</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HBV/HCV/AIH/PBC/</td>
<td>3/0/2/0</td>
<td>7/1/3/0</td>
<td>2/8/2/1</td>
<td>7/15/0/0</td>
<td>—</td>
<td></td>
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<tr>
<td>Alcohol/Drug/NAFLD/</td>
<td>4/3/0</td>
<td>4/3/0</td>
<td>6/0/2</td>
<td>1/1/0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>0/2</td>
<td>4/2</td>
<td>3/0</td>
<td>0/0</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Applicable values are presented as means ± SD or median[range]. AST, aspartate aminotransferase; ALT, alanine aminotransferase; Plt, platelets; PT, prothrombin time; PT-INR, prothrombin time and international normalized ratio; HBV, hepatitis B virus; HCV, hepatitis C virus; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; NAFLD, nonalcoholic fatty liver disease.

SPF conditions, and all the experiments using animals were performed under institutional guidelines. Mouse liver progenitor cells were induced by feeding 8-wk-old male mice a diet containing 0.1%, 3.5-diethoxycarbonyl-1,4-dihydroludeline (DCC) (CLEA). In a gene-delivery experiment involving hydrodynamic tail vein injection (HTVi), male mice weighing 18–20 g were used. The experiments involving both gene delivery and animals were approved by the institutional review board (animal experiment; approval number 24139, gene-splicing research; approval number 0.88).

**Gene delivery.** A plasmid in which mouse epiregulin cDNA was cloned into the pLIVE (Liver in Vivo Expression) vector and the empty expression pLIVE vector (Mirus Bio, Madison, WI) were purchased from Takara (Shiga, Japan). The mouse epiregulin plasmid was amplified by Escherichia coli DH5α competent cells (Takara) and purified using an Endofree Plasmid Mxi Kit (Qiagen, Düsseldorf, Germany). Plasmid DNA was administered to mice using a HTVi technique involving rapid injection of a large volume of DNA solution through the tail vein. Briefly, 20 μg of plasmid DNA (1 mg/ml) was diluted in 2 ml of TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio) and injected into male mice weighing 18–20 g via the tail vein within 6 to 8 s.

**Immunohistochemistry.** For immunohistochemistry (IHC), a rabbit polyclonal antibody against mouse CK19 (Proteintech, Chicago IL) and a rabbit polyclonal antibody against mouse epiregulin (Abbiotec, San Diego, CA) were used at a dilution of 1:50. A mouse monoclonal antibody against PCNA (clone PC10; Nichirei, Tokyo, Japan), a rat monoclonal antibody against mouse epithelial cell adhesion molecule (EpCAM) (BD Pharmingen, San Diego, CA), and a rat monoclonal antibody against mouse Thy1 (BD Pharmingen) were used at a dilution of 1:100. The formalin-fixed paraffin-embedded liver sections (3 μm) underwent antigen retrieval processing in a microwave oven after being blocked with one Histo reagent (Nakarai, Kyoto, Japan), the samples were incubated with primary antibodies and subsequently with high-molecular-weight polymer secondary antibodies (Simple Stain kit, Nichirei). The samples were finally developed with a peroxidase substrate ImmPACT NovaRED kit (Vector Laboratories, Burlingame, CA). Frozen sections (6 μm) from the liver were placed on APS-coated glass slides using a cryostat (Leica CM 3050S). After being fixed with ethanol, the samples were incubated with the primary antibodies and then incubated with fluorescence-conjugated secondary antibodies before observation by fluorescence microscopy (D1; Zeiss Axios Observer, Jena, Germany).

**RNA extraction and RT-PCR.** Total RNA was extracted from cells and tissues using a RNasy Plus Mini Kit (Qiagen). Total RNA (2 μg) was used to synthesize cDNA using a SuperScript VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA). The samples were subjected to 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. PCR primers for mouse genes were as follows (5’ to 3’): l) epiregulin, TGGGGTCTT-GACGCTGTTTGTG and TGGAGTCACTCTCATAATTC; 2) EGFR, CTGCCCAAGGCAACAGTAACA and ATTTGGAAGCTTGGTGTG; 3) Erbb4, GCTGGAGAATATGGTGTGCCCGAG and AAA-CATCTCAGGCTTGACCCCTG; 4) β-actin, GCCTGGATATCTCCATCG and CCAGTGGTAATGCGATGT. Quantitative real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) with the TaqMan Gene Expression Assay (Assay ID: epiregulin, Mm00514794_m1; and GAPDH, Mm99999915_g1).

**Cell culture and proliferation assay.** An EpCAM-positive liver progenitor cell line from the liver of a DDC-fed adult mouse was kindly provided by Dr. Atsushi Miyajima (Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo). The EpCAM+ cells were maintained in a cell culture flask (Corningware, Corning, NY) using medium supplemented with fetal bovine serum and 10 ng/ml of each human recombinant EGF and hepatocyte growth factor (HGF). The proliferative response of EpCAM+ cells was exam...
Mouse liver progenitor cells (LPCs) were induced by a diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) in C57BL/6J mice. A: liver turned black and stiff after 5 wk on the DDC diet. B: hematoxylin and eosin (HE) staining of livers from mice on the DDC diet demonstrated ductular proliferation consisting of small cells in the portal area from 1 to 5 wk. These small cells were positive for CK19, indicating that they were LPCs. The expression of epiregulin protein was markedly elevated around ductular structures close to the portal area during the course of DDC-induced liver injury. Bars = 100 μm.
ined in the presence of FBS, EGF, or HGF in different concentrations, using the water-soluble tetrazolium salt (WST-1) cell proliferation reagent (Roche, Basel, Switzerland). The absorbance value (OD450-OD650) was measured using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA).

Serum epiregulin immunoassay. The human and mouse serum epiregulin values were measured using an epiregulin-specific ELISA (USCN, Hubei, China) in accordance with the manufacturer’s protocol (assay range: 6.4–1,000 pg/ml). The absorbance value (450 nm) was measured using a Benchmark Plus microplate reader (Bio-Rad).

Statistical analysis. Comparison of gene expression assays, immunonoassay, and patient background were done using one-way ANOVA with subsequent Steel-Dwass or Tukey-Kramer tests. Analysis of the cell proliferation assay was done using two-way ANOVA. Differences at \( P < 0.05 \) were considered to be statistically significant. The above statistical analyses were done using the SAS software package (SAS Institute, Cary, NC).

RESULTS

Elevation of serum epiregulin levels in patients with acute liver failure. To investigate whether epiregulin is involved in liver regeneration, we determined the serum epiregulin levels in patients with a variety of liver diseases, including acute liver failure, acute hepatitis, chronic hepatitis, and liver cirrhosis. Those in healthy subjects were also examined for comparison. Acute liver failure was defined as severe liver dysfunction with a prothrombin time of less than 40% or prothrombin time and international normalized ratio (commonly called PT-INR) > 1.5 within 8 wk after initial presentation (14). There was no significant difference in background factors such as age or sex ratio between the groups. Other characteristics including the degree of liver damage are listed in Table 1. Serum epiregulin levels in patients with acute liver failure were significantly higher than in healthy controls (Fig. 1). However, there were no significant differences among the other disease groups. This observation may have reflected the promotion of epiregulin production only under conditions in which proliferation of normal hepatocytes would be limited.

Expression of epiregulin in injured adult mouse liver concomitant with LPC emergence. The 2-acetylaminofluorene (2-AAF)/PH model, in which hepatocyte proliferation is blocked by 2-AAF before PH, has been used to induce LPCs in rats (5, 6). Recently, a 0.1% DDC-containing diet has been developed to induce LPCs in mice, and this mouse model was employed for the present study (1, 17). After 4–5 wk on the DDC diet, the liver of mice appeared black and stiff (Fig. 2A). Hematoxylin and eosin staining was used to evaluate adult normal liver and liver with DDC-induced injury. After 1 wk on the DDC diet, the liver demonstrated ductular structures around the portal area, and these gradually increased up to 5 wk (Fig. 2B). These ductular structures consisted of small cells with large nuclei, with a so-called “oval cell” appearance. IHC for CK19, one of the markers of LPCs and cholangiocytes, showed that these small cells included CK19-expressing LPCs (Fig. 2B). Epiregulin protein expression was barely detectable in normal liver but was markedly induced around the ductular structures close to the portal area during the course of DDC-induced liver injury (Fig. 2B). Quantitative gene expression analysis confirmed that the expression of epiregulin mRNA was significantly increased in the DDC-injured liver after 4–5 wk on the DDC diet (Fig. 3, A and B). In addition, the serum epiregulin level was significantly increased from 2 wk on the DDC diet and gradually increased up to 4 wk (Fig. 3C).

Coexpression of epiregulin with Thy1-positive cells around ductular structures in injured mouse liver. To investigate the site of epiregulin expression, we examined injured mouse liver using IHC. As briefly mentioned above, IHC for CK19 and epiregulin confirmed that epiregulin was expressed around the LPCs forming ductular structures. Recently, as it has been reported that Thy1-positive cells express growth factors nec-
necessary for the maintenance of LPCs (15), we performed IHC for epiregulin and Thy1, which are markers of mesenchymal cells or LPCs. First, we performed double immunostaining for CK19 and EpCAM as LPC markers and confirmed that both were coexpressed around ductular structures in the portal area at 5 wk. Double immunostaining for epiregulin and EpCAM revealed that epiregulin was expressed adjacent to the LPCs forming ductular structures. Further double immunostaining for Thy1 and epiregulin revealed partial coexpression of these two markers (Fig. 4).

Epiregulin promotes the proliferation of EpCAM+ LPCs in vitro. Because epiregulin was expressed close to LPCs in injured mouse liver, we performed an in vitro study to investigate whether epiregulin affects LPCs. First, to determine whether epiregulin acts on LPCs directly, we examined the expression of the epiregulin receptors EGFR and ErbbB4 in EpCAM+ LPCs. Although epiregulin is known to bind to and activate both EGFR and ErbbB4 (11), only EGFR was expressed in EpCAM+ LPCs (Fig. 4A). Subsequently, we examined the proliferation of LPCs under different dosages of either recombinant epiregulin or EGF, another EGFR ligand. EpCAM+ LPCs were exposed to 0, 20, or 100 ng/ml epiregulin or EGF in culture, and their proliferations were measured after 3 days of incubation in 96-well plates using the standard WST-1 assay. This revealed that recombinant epiregulin promoted the proliferation of EpCAM+ LPCs in a concentration-dependent manner, and the proliferation efficacy was significantly higher in epiregulin than EGF (Fig. 4B).

Epiregulin overexpression in adult mouse liver induces LPCs and promotes the synthesis of DNA in hepatocytes. To investigate the effect of epiregulin on hepatocytes, the epiregulin gene was overexpressed in mice using the HTVi method, which involves injection of plasmid DNA via a tail vein and facilitates overexpression of the target gene in organs including the liver. Since Zhang et al. (19, 31) first reported the HTVi method in 1997, it has been used in various gene overexpression studies because of its 1) high transfer efficiency and reproducibility and 2) easier preparation of plasmid DNA (30). The epiregulin-overexpressing mice were killed at 3, 7, 14, and 21 days after gene delivery. The control mice were injected with empty expression plasmid vector. IHC for epiregulin confirmed its expression in the entire liver up to 21 days after gene delivery, reaching a peak during the initial 3–7 days (Fig. 5A). Empty vector-injected mice livers demonstrated no detectable levels of epiregulin expression. To evaluate DNA synthesis, IHC for PCNA was performed on epiregulin-overexpressing mouse liver. IHC for CK19 confirmed that LPC induction was evident from 3 days after epiregulin gene delivery (Fig. 5A). Furthermore, the ratio of PCNA-positive cells was increased significantly at 3, 7, and 14 days after epiregulin gene delivery (Fig. 6, A and B).

**DISCUSSION**

In the present study, we have shown that expression of epiregulin promoted the induction of LPCs and DNA synthesis by hepatocytes and that epiregulin was expressed by the stem cell niche including mesenchymal cells located around ductular structures in the portal area of adult mice. Furthermore, the serum epiregulin level was elevated in both patients and mice with liver injury. Taken together, these data suggested that

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**Fig. 4.** Double immunostaining study of mouse liver after DDC diet feeding. First, we performed double immunostaining for CK19 and epithelial cell adhesion molecule (EpCAM) as LPC markers and confirmed that both markers were coexpressed and associated with ductular structures in the portal area of mice at 5 wk. Double immunostaining for epiregulin and EpCAM revealed that epiregulin was expressed adjacent to LPCs forming ductular structures. Further double immunostaining for Thy1 and epiregulin revealed partial coexpression of these 2 markers. Bar = 200 μm.
Epiregulin was expressed from 1 wk on the DDC diet and then gradually increased, reaching a peak at 4–5 wk. This course of expression was identical to that of CK19+ LPCs. We have also shown that epiregulin was expressed in the area of LPCs forming ductular structures, i.e., the so-called stem cell niche, and was partially coexpressed by Thy1-positive cells. It has been reported that immune cells or mesenchymal cells emerge around the LPCs (10, 21). Recently, FGF7, another growth factor, was shown to be expressed by Thy1-positive cells forming the stem cell niche and to contribute to the maintenance of liver regeneration (15). Taken together, the data suggest that epiregulin may also contribute to liver regeneration through expression by Thy1-positive cells, which belong to the LPC niche. However, because epiregulin was partially coexpressed with Thy1, other candidate epiregulin-producing cells may exist. In fact, heparin-binding-EGF, a hepatocyte growth-promoting factor belonging to the EGF family, is expressed by both endothelial cells and Kupffer cells (9).

In our in vivo study of mice with epiregulin overexpression, we have confirmed that epiregulin promoted the synthesis of DNA by mature hepatocytes and did not affect LPCs alone. The present study is the first study describing the ability of epiregulin to promote DNA synthesis in vivo. EGF was originally characterized as a growth factor that binds to specific receptors on the cell surface, stimulating protein tyrosine kinase activity leading to cellular proliferation and DNA synthesis (3, 4, 29). However, the signaling potencies of epiregulin and EGF were considered to be different (12). In particular, epiregulin is reported to exhibit modest activating effects for EGFR and MAPK phosphorylation although activating effects of epiregulin were supposed to be prolonged compared with those of EGF (12). In our in vitro study, epiregulin promoted the proliferation of LPCs, and its efficacies were higher than EGF. This biological difference may be derived partially due to the weak activating capabilities of epiregulin, which in turn affected to prevent the negative downstream regulations.

In this study, although epiregulin was shown to promote the induction and proliferation of LPCs, further analysis will be needed to confirm whether epiregulin is really essential for LPC induction, perhaps employing epiregulin gene-knockout mice. Although epiregulin gene-knockout mice have already been established, there have been no reports indicating that they have developmental anomalies in the liver (20). Using these epiregulin-knockout mice fed a DDC diet, we were able to evaluate whether LPCs were induced. Further analyses will be needed to determine whether there are unspecified epiregulin-expressing cells that are Thy1 negative.

In conclusion, epiregulin 1) is expressed in the stem cell niche, which includes mesenchymal cells around the LPCs during severe liver injury, 2) contributes to liver regeneration by inducing LPCs, and 3) promotes DNA synthesis by mature hepatocytes. In addition, the serum epiregulin level is significantly elevated in both patients and mice with liver injury. Taken together, these data indicate that epiregulin would be a useful biomarker of liver regeneration.

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Fig. 6. The epiregulin gene was overexpressed using the hydrodynamic tail vein injection method in C57BL/6J mice. A: immunohistochemistry for epiregulin confirmed expression of the protein from 3 to 21 days after gene delivery. Control mice were studied 3 days after injection with empty expression plasmid vector. Along with epiregulin expression, the ratio of PCNA-positive cells was significantly increased at 3 to 14 days. In addition, ductular proliferation consisting of CK19-positive LPCs was confirmed at 3 to 21 days. Bar = 200 μm. B: ratio of PCNA-positive cells relative to the value in normal mice. The PCNA positivity ratio was significantly increased up to 14 days and peaked at 3 days after gene delivery. Means ± SD, **P < 0.01.

GRANTS
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES