Transit-Amplifying Ductular (Oval) Cells and Their Hepatocytic Progeny Are Characterized by a Novel and Distinctive Expression of Delta-Like Protein/Preadipocyte Factor 1/Fetal Antigen 1

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Hepatic regeneration from toxic or surgical injury to the adult mammalian liver, endorses different cellular responses within the hepatic lineage. The molecular mechanisms determining commitment of a cell population at a specific lineage level to participate in liver repair as well as the fate of its progeny in the hostile environment created by the injury are not well defined. Based on the role of the Notch/Delta/Jagged system in cell fate specification and recent reports linking Notch signaling with normal bile duct formation in mouse and human liver, we examined the expression of Notch1, Notch2, Notch3, Delta1, Delta3, Jagged1, and Jagged2, and delta-like protein/preadipocyte factor 1/fetal antigen 1 (dlk) in four well-defined experimental rat models of liver injury and regeneration. Although Delta3 and Jagged2 were undetectable by reverse transcriptase-polymerase chain reaction and Northern blot, we observed the most significant up-regulation of all other transcripts in the 2-acetylaminofluorene-70% hepatectomy (AAF/PHx) model, in which liver mass is restored by proliferation and differentiation of transit-amplifying ductular (oval) cells. The most profound change was observed for dlk. Accordingly, immunohistochemical analyses in the AAF/PHx model showed a specific expression of dlk in atypical ductular structures composed of oval cells. Delta-like protein was not observed in proliferating hepatocytes or bile duct cells after partial hepatectomy or ligation of the common bile duct whereas clusters of dlk immunoreactive oval cells were found in both the retrorsine and the AAF/PHx models. Finally, we used dlk to isolate α -fetoprotein-positive cells from fetal and adult regenerating

rat liver by a novel antibody panning technique. (*Am J Pathol* 2004, 164:1347-1359)

In certain types of toxic hepatic injury impairing the replication of hepatocytes, transit-amplifying populations of ductular cells with an oval-shaped nucleus and a high nuclear to cytoplasmic ratio, are produced. The result is an intricately intertwined network of ductular structures with a poorly defined lumen (ie, atypical ductular reactions) radiating from the periportal region into the parenchyma. The transit-amplifying ductular (oval) cells share some phenotypic characteristics with the bipotential fetal hepatoblasts and may, if needed, differentiate to hepatocytes or bile duct cells and reconstitute the architecture and function of the damaged liver tissue.¹⁻⁴ Although the origin of oval cells has not been conclusively established, evidence points to endogenous stem cells located at the junctions between bile duct cells and hepatocytes in the terminal bile ductules (the canal of Hering) as a potential source.⁴⁻⁶

It is also well established that reconstruction of liver mass lost to surgical resection is accomplished by proliferation of residual, normally quiescent hepatocytes and bile duct cells responding rapidly and giving rise to a large number of progeny while maintaining their differentiated phenotype.^{7,8} Furthermore, regeneration in response to other types of toxic hepatic injury impairing hepatocyte replication appears to be accomplished by vigorously proliferating small hepatocyte-like progenitor cells expressing phenotypic characteristics of fetal hepatoblasts and adult mature hepatocytes.^{9,10} Therefore, the tremendous capacity for hepatic regeneration may result from the ability to call forth a cellular response at different

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levels in the hepatic lineage. This has led to the hypothesis that similar to other organs the cellular lineage of the liver consists of true endogenous stem cells, progenitor cells (ie, oval cells and hepatocyte-like progenitors), and mature differentiated cells (hepatocytes and bile duct cells).¹¹ However, recent evidence also indicates that two sources of stem cells may be called on to participate in liver regeneration: endogenous stem cells located in the canal of Hering and exogenous stem cells derived from the bone marrow and capable of differentiation into hepatocytes and bile duct cells on homing to the injured liver.^{12,13}

The details regarding the molecular mechanisms that determine the commitment of a cell population at a specific lineage level to participate in liver repair as well as the fate of its progeny in the hostile environment created by the injury still remains to be elucidated. One well-characterized example of a highly conserved mechanism of cell fate specification playing a pivotal role in vertebrate development is the lateral protein-protein interaction in the Notch-Delta or Notch-Jagged/Serrate systems. These proteins all belong to the epidermal growth factor (EGF)-like homeotic protein family, the members of which are characterized by the presence of EGF-like motifs. Upon interaction of the Notch receptor with its ligands Delta or Jagged/Serrate, the receptor is processed by proteolysis and the subsequent nuclear translocation of the receptor's intracellular domain results in transcription of lineage-specific genes.¹⁴ In vertebrates, a diverse repertoire of Notch-related molecules has been identified. Notch itself comprises a family of single pass transmembrane proteins with four homologs, Notch1, Notch2, Notch3, and Notch4.^{15–17} The Notch ligands are also transmembrane proteins and belong to the DSL (Delta, Serrate, Lag-2) family of proteins. All ligands are characterized by two conserved motifs: the DSL domain, important for Notch binding and a series of EGF-like repeats. They are grouped into two subfamilies based on the presence of a cysteine-rich region in the extracellular region: those with the cysteine-rich region belong to the Serrate/Jagged family, whereas those without belong to the Delta family. The intracellular domains are poorly conserved, suggesting that the primary function of the ligands is to activate Notch through well-conserved extracellular domains. Identified initially in invertebrates, four members of the Delta family, Delta1, Delta2, Delta3, and Delta4,^{18–20} and two of the Jagged/Serrate family (Serrate is the *Drosophila* homologue), Jagged1 and Jagged2,^{21,22} have been characterized in mammals thus far. Notch function has been studied extensively in mouse models, in which it is involved in early pattern formation of the embryo,^{23,24} as well as stem cell fate specification in brain, skin, pancreas, and hemopoietic tissue.^{25–29}

Delta-like protein (dlk) also belongs to the EGF-like homeotic protein family. Delta-like protein is a transmembrane protein containing six EGF-like repeats, a signal sequence in the extracellular domain, a unique transmembrane domain, and a short intracellular region.^{30,31} Delta-like protein, also referred to as pG2 (a human adrenal-specific mRNA), Pref-1 (preadipocyte factor 1),

SCP-1, ZOG, as well as the soluble product FA1 (fetal antigen 1)³² are encoded by the gene *DLK1*. The function of dlk is not known but the protein is widely expressed in embryonic tissues, including liver, tongue, vertebrae, skeletal myotubes, chondroblasts, and pancreas,^{33,34} whereas in adults it is confined to the zona glomerulosa of the adrenal gland,³² the somatotrophs in the pituitary gland,³⁵ the monoaminergic neurons in the central nervous system,³⁶ the Leydig and hilus cells of the testes and ovaries,³⁷ and the β cells in the endocrine pancreas.³³ The cysteine spacing and the amino acid sequence within the individual EGF-like repeats are similar to those of Delta, which contains nine EGF-like repeats and the cysteine-rich motif DSL important for binding to Notch in the extracellular region. However, the DSL motif is absent from dlk, indicating that dlk is an unlikely ligand for Notch. Therefore, the organization of the *DLK1* gene, and the structure of the individual EGF-like repeats strongly suggest that dlk is most closely related to the Delta family. Similar to the Notch-Delta or Notch-Jagged/Serrate systems, dlk may play a role in cell-to-cell interactions controlling cell fate determination during embryonic development and in the adult organism.³⁸

Tissue localization studies of Notch receptor and ligand expression in adult human liver have suggested that Notch signaling may be important for normal bile duct formation.^{39,40} Delta-like protein is highly expressed in human and mouse fetal liver where it localizes to the hepatoblasts with the biliary epithelial and hematopoietic cell compartments showing no expression.^{34,41} However, a thorough analysis of the role of these developmentally important proteins in regulating the commitment of liver progenitor cells to participate in liver repair as well as in determining their fate in a hostile environment created by liver injury in the adult organism has not been performed. In the present study we have, using a number of well-defined experimental rat models of liver injury and proliferative regeneration, examined the expression of Notch1, Notch2, Notch3, Delta 1, Delta3, Jagged1, Jagged2, and dlk in cell populations at different levels of differentiation within the cellular lineage of the liver.

Materials and Methods

Animal Models

Male Wistar rats, 8 weeks of age, were purchased from Taconic M&B (Ry, Denmark) and kept under standardized conditions with access to food and water *ad libitum*. Four experimental models were used to induce cell proliferation at different levels within the hepatic lineage: 1) proliferation of mature hepatocytes and bile duct cells was accomplished by surgical resection of the median and left lateral liver lobes removing ~70% of the liver mass (PHx protocol)⁸; 2) proliferation and differentiation of transit-amplifying ductular (oval) cells was achieved through treatment with 2-acetylaminofluorene (2-AAF) (9 days, 20 mg/kg/day by gavage) interrupted at day 5 to perform a 70% hepatectomy (AAF/PHx protocol)⁴²; 3) proliferation of small hepatocyte-like progenitor cells was

initiated by two intraperitoneal injections of retrorsine (30 mg/kg) 2 weeks apart, followed by a 70% hepatectomy 5 weeks after the last injection of retrorsine (retrorsine/PHx protocol)⁹; and 4) proliferation of mature bile duct cells was induced by ligation of the common bile duct for 5 days.⁴³ Controls included: 1) a sham operation with laparotomy only; 2) treatment with 2-AAF as above except that the 70% hepatectomy was replaced by a sham operation; and 3) treatment with retrorsine as above with the 70% hepatectomy replaced by a sham operation. Groups of three animals were sacrificed by cervical dislocation as indicated in the figures and parts of the liver were snap-frozen in liquid nitrogen for extraction of total RNA (RNeasy kit; Qiagen Inc., Santa Clarita, CA), or fixed for histological examination. The Danish Council for Supervision with Experimental Animals had approved usage of the models.

Cell Isolation

Rat hepatoblasts were isolated from embryos at day 21 after gestation. After excision, the livers were washed once in phosphate-buffered saline (PBS) without calcium and magnesium, minced in Ham's F12/Dulbecco's modified Eagle's medium (with Glutamax and pyridoxine; Invitrogen Corp., Paisley, Scotland) supplemented with 10 mmol/L HEPES and 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO), and incubated at 37°C for 45 minutes with intermittent shaking. The cell suspension was diluted in Ham's F12/Dulbecco's modified Eagle's medium supplemented with 10 mmol/L HEPES, 10% fetal bovine serum (BIOCHROM KG, Berlin, Germany), and 50 µg/ml gentamicin, filtered through a nylon cloth and pelleted by centrifugation. Subsequently, the cell pellets were washed twice and resuspended in Ham's F12/Dulbecco's modified Eagle's medium supplemented with 10 mmol/L HEPES and 50 µg/ml gentamicin. Typically, more than 80% of the hepatoblasts excluded trypan blue.

Nonparenchymal cell populations were isolated from adult rat liver by a two-step perfusion technique. Rat livers were perfused *in situ* for 15 minutes with Hanks' balanced salt solution without calcium and magnesium (500 ml, 30 ml/minute), followed by 0.2% pronase (200 ml, 30 ml/minute); protease from *Streptomyces griseus* (Sigma-Aldrich) dissolved in MEM (Invitrogen), and finally 1% pronase, 0.1% collagenase type I (Worthington Biochemical Corp., Freehold, NJ) and 0.007% DNase I (Worthington) in MEM (50 ml, 30 ml/minute). The liver was excised, minced, and incubated in the latter medium at 37°C for 60 minutes with intermittent shaking. The cell suspension was filtered through a nylon cloth, diluted in Williams E medium (Invitrogen) containing 10% fetal bovine serum and 50 µg/ml gentamicin, and the cells sedimented by centrifugation. The pellets were washed four times and purified on a two-step Percoll gradient (30% and 70% Percoll; Sigma-Aldrich). After application of the cell suspension onto the upper cushion, the gradient was centrifuged at 800 × g for 45 minutes at 20°C. The band of cells localized between the two Percoll cushions was collected, the cells sedimented by centrifugation,

washed once in Williams E medium containing 10% fetal bovine serum and 50 µg/ml gentamicin, and suspended in the latter medium. When visualized by light microscopy less than 1% of the cells were hepatocytes according to their size, and typically more than 95% of the cells excluded trypan blue.

Cell Sorting and Culture

Cell sorting of dlk-expressing cell populations were achieved using a novel functional antibody surface.⁴⁴ In brief, an anthraquinone conjugate (Exiqon, A/S, Vedback, Denmark EQ-0051-10; Denmark) consisting of an anthraquinone molecule, a linker, and an electrophilic group, was diluted in sterile water (1:3000), and applied to 55-mm bacteriological polystyrene Petri dishes. The dishes were UV irradiated at 340 nm for 10 minutes using an ULS-20-2 illuminator (Philips Cleo Compact 25W-S; UV-Lights Systems, Denmark). After washing the dishes three times with sterile water, a polyclonal rabbit anti-rat FA1 antibody (monospecific rabbit anti-rFA1, 0.9 mg/ml affinity purified) diluted 1:3000 or 1:6000 in 100 mmol/L sodium phosphate buffer (pH 8.0) was applied on the surface. This antibody was generated using affinity-purified rat FA1 from amniotic fluid representing the large soluble product containing the extracellular domain of dlk. The dishes were incubated for 2 hours at room temperature with gentle agitation and washed three times with PBS containing 0.05% Tween 20 to remove excess antibody. Control dishes without antibody were prepared in parallel.

For cell adherence, isolated fetal cells or adult nonparenchymal liver cells were applied to the antibody-coated Petri dishes and incubated for 60 minutes at 37°C (CO₂ atmosphere). Cells that did not adhere to the dishes were removed by washing the dishes three times in PBS and collected by centrifugation for RNA extraction. For cell culture, the Petri dishes with the adhering cells were overlaid with Ham's F12/Dulbecco's modified Eagle's medium (with Glutamax and pyridoxine) supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, and 50 µg/ml gentamicin and cultured for 5 days. Total RNA was extracted from the adhering cell populations and the collected nonadhering cell pellets using Trizol reagent (Invitrogen).

Northern Blot Analysis and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Northern blot analysis was performed by electrophoresis of 10 µg of total RNA in 1% agarose/0.2 formaldehyde gels and transfer onto nylon membranes. For quantification, a slot blot analysis was performed by immobilizing 5 µg of total RNA onto nylon membranes. Membranes were hybridized to cDNA probes labeled with [³²P]dCTP. The cDNA probes were generated by RT-PCR, cloned into the pCRII plasmid vector (TA cloning kit, Invitrogen), and sequenced to verify their identity. The rat α-fetoprotein

(AFP) cDNA encompassed nucleotides 101 to 329 (GenBank accession no. X02361); rat dlk nucleotides 1 to 1152 (GenBank accession no. U25680); rat Jagged1 nucleotides 387 to 1073 (GenBank accession no. NM_019147); rat Jagged2 nucleotides 1 to 870 (GenBank accession no. U70050); rat Delta1 nucleotides 290 to 2434 (GenBank accession no. U78889); rat Delta3 nucleotides 19 to 1796 (GenBank accession no. AF084576); rat Notch1 nucleotides 241 to 1060 (GenBank accession no. X57405); rat Notch2 nucleotides 511 to 1320 (GenBank accession no. M93661); rat Notch3 nucleotides 94 to 904 (GenBank accession no. AF164486). Bound radioactivity was detected and quantified using a STORM 840 PhosphorImager (Molecular Dynamics). The filters were hybridized with rat S18 to assess the integrity and ensure equal loading of the RNA.

For RT-PCR analysis, first strand complementary DNA (cDNA) was prepared from 1 μ g of total RNA using the Reverse Transcription System (Promega Corp., Madison, WI) and random primers. The cDNAs were normalized for equivalent template amounts by amplification with primers for rat TATA binding protein (TBP) and the LightCycler Instrument (Roche a/s Applied Science, Hvidovre, Denmark) using a SYBR Green PCR protocol. The cDNAs then provided templates for a semiquantitative PCR using Elongase Enzyme Mix (Invitrogen) and the following forward and reverse primers: rat AFP 5'-agactcttcccaatgccgacagag-3' and 5'-gggtctcttcgacacatcgtagatg-3'. After an initial denaturation step at 94°C for 5 minutes, 40 cycles at the following parameters were performed: 94°C for 30 seconds, 65°C for 30 seconds, and 68°C for 3 minutes. Finally, an extension step at 68°C for 7 minutes was performed. The PCR products were separated on a 1.2% agarose gel and visualized by SYBR Green II (Sigma-Aldrich).

Immunohistochemistry

Specimens fixed in formalin and embedded in paraffin were cut in 5- μ m sections, allowed to air dry, and subsequently deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with H₂O₂/methanol, and endogenous biotin with a biotin blocking kit (X0590; DAKO A/S, Glostrup, Denmark). Antigen retrieval for rat dlk was performed by incubation with 0.05% (w/v) protease (type XIV, Sigma-Aldrich) in Tris-buffered saline at 37°C for 15 minutes. Demasking of the Notch1, Notch2, Notch3, Delta1, Jagged1, hdlkC, and proliferating cell nuclear antigen (PCNA) was done by microwave heating three times, 5 minutes each (at 800 W, 400 W, and 400 W, respectively) in TEG buffer (10 mmol/L Tris, 0.5 mmol/L EGTA, pH 9). For detection of rat dlk, the primary antibody (monospecific rabbit anti-rFA1, see Cell Sorting and Culture section) or control antibody (primary antibody liquid phase absorbed with affinity-purified rat FA1) was diluted 1:2000. An anti-hdlkC antibody was produced by affinity purification of serum from rabbits immunized with a fusion protein containing the entire intracellular region of human dlk³⁰ and was used in a 1:300 dilution. This antibody reacts with the intracellular part of rat and hu-

man dlk. Polyclonal goat antibodies directed against Delta1 (sc-8155, diluted 1:400), Jagged1 (sc-6011, diluted 1:500), and Notch1 (sc-6014, diluted 1:150) and polyclonal rabbit antibodies directed against Notch2 (sc-5545, diluted 1:150) and Notch3 (sc-5593, diluted 1:1000) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the monoclonal anti-PCNA (M879, diluted 1:400) from DAKO. The secondary antibodies [biotinylated goat anti-rabbit Ig (E432, diluted 1:200), biotinylated goat anti-mouse Ig (E433, diluted 1:200), and biotinylated rabbit anti-goat Ig (E466, diluted 1:300)] were also obtained from DAKO. After incubations with primary and secondary antibodies, sections were incubated with horseradish peroxidase-conjugated streptavidin (P397, DAKO) diluted 1:300 and developed using 3-amino-9-ethylcarbazol as chromogen. Counterstaining of nuclei was performed with hematoxylin.

Results

Expression Profiles of Notch1, Notch2, Notch3, Delta1, Delta3, Jagged1, Jagged2, and Dlk in Regenerating Liver

To investigate if Notch signaling could be a mechanism for cell fate specification within the hepatic lineage, Northern blot analysis was performed on liver regenerating by 1) replication of existing mature hepatocytes and bile duct cells after a partial hepatectomy, 2) amplification and differentiation of oval cell populations using the AAF/PHx protocol, and 3) proliferation of small hepatocyte-like progenitor cells using the retrorsine/PHx protocol. Protocols in which a sham operation replaced the partial hepatectomy served as the reference models. Levels of the three Notch receptors did not show significant changes in gene expression throughout a 9-day period after regeneration was initiated when mature hepatic cells (the PHx protocol) or small hepatocyte-like progenitor cells (Ret/PHx protocol) were involved (Figure 1). Similarly, no changes were seen in the corresponding sham protocols. However, small but detectable changes were observed at day 5 and day 9 after PHx in the AAF/PHx protocol at a time when oval cells transiently proliferate and invade the hepatic parenchyma. Similarly, expression changes of the Notch receptor ligands Delta1 and Jagged1 were also most pronounced in the AAF/PHx protocol with Jagged1 showing the most significant changes in expression levels. For the ligands Delta3 and Jagged2, expression in liver was not detected by RT-PCR or Northern blot analysis. The most remarkable changes in expression profiles were detected for dlk and AFP (an established marker for oval cells and hepatoblasts) showing high expression levels at day 9 after PHx in the AAF/PHx protocol. In Figure 1, only RNA from one animal of a group of three was run on the Northern blot. However, a slot blot analysis of all animals included in the study confirmed the expression profiles of the Notch receptors, their ligands, dlk, and AFP (data not shown).

Figure 1. Gene expression analysis by Northern blotting. Each lane contains 10 μ g of total RNA isolated from one rat liver. Membranes were hybridized to cDNA probes labeled with [32 P]dCTP and visualized by autoradiography. Hybridization with S18 was used to assess integrity and equal loading of RNA samples. AAF/sham, treatment with 2-AAF and laparotomy only; Ret/sham, treatment with retrorsine and laparotomy only; sham, laparotomy only; AAF/PHx, treatment with 2-AAF and a 70% hepatectomy; Ret/PHx, treatment with retrorsine and a 70% hepatectomy; PHx, 70% hepatectomy only. Animals were sacrificed 1, 5, and 9 days after the surgical procedure.

Cellular Localization of Notch, Delta, and Jagged/Serrate Proteins in Regenerating Liver

To localize the expression of the Notch receptors and the ligands Delta1 and Jagged1 within the adult regenerating liver, immunohistochemical analysis was performed on liver tissue sections. The most pronounced changes in protein expression patterns were observed in liver sections obtained from animals treated according to the AAF/PHx protocol at day 9 after the partial hepatectomy. These changes are illustrated in Figure 2. In the liver responding to a sham operation, we observed a normal morphology with a few cells within the bile ducts as well as a few hepatocytes in the hepatic parenchyma showing nuclear staining for PCNA (in the present study used as a marker for cellular proliferation) (Figure 2A). This pattern of cell proliferation was identical to the pattern observed in liver sections from control animals not subjected to any treatment protocols (data not shown). Therefore, liver tissues from sham-operated animals were used as the appropriate controls in the present study, and will in the

following be referred to as control liver. In control liver, Notch1 immunoreactivity was present exclusively in bile ducts, the endothelium of hepatic arteries and occasionally in sinusoidal endothelial cells (Figure 2B). Weak Notch2 staining was found on bile duct cells and hepatocytes whereas the endothelium of hepatic arteries and veins were negative (Figure 2C). Strong Notch3 immunostaining decorated bile duct cells, membranes of hepatocytes, and the endothelium of the hepatic veins (Figure 2D). Immunostaining for Delta1 revealed a weak expression only in hepatocytes (Figure 2E) whereas Jagged1 expression was observed in bile duct cells and to a lesser extent in hepatocytes as well as in the endothelium of hepatic arteries and veins (Figure 2F).

In liver regenerating at day 9 after PHx in the AAF/PHx protocol a large number of transit-amplifying cells with nuclear PCNA immunostaining was observed (Figure 2I). The PCNA-positive cells were arranged in ductular structures with a poorly defined lumen extending from the portal area into the hepatic parenchyma. The nuclei in mature hepatocytes and well-defined bile ducts did not stain. Weak immunoreactivity of Notch1 was found in mature hepatocytes and some sinusoidal endothelial cells whereas oval cells and bile duct cells were negative (Figure 2J). For Notch2 a weak immunoreactivity was observed in all hepatic cells except the endothelium of the hepatic veins. A stronger reactivity was observed in some, possibly inflammatory, cells (Figure 2K). A strong Notch3 immunoreactivity was localized in the membranes of hepatocytes while staining in the oval cells and bile duct cells was absent (Figure 2L). Delta1 expression was detected in some mature hepatocytes whereas oval cells and bile duct cell were negative (Figure 2M). In contrast, Jagged1 expression was detected in oval cells, bile duct cells, and occasionally in mature hepatocytes (Figure 2N).

We also performed immunohistochemistry on liver sections from rat liver regenerating in response to a partial hepatectomy (PHx protocol) and to treatment with retrorsine combined with a partial hepatectomy (Ret/PHx protocol). The results of these studies with respect to the epithelial cell compartments in the various regenerative models are summarized in Table 1. The most pronounced changes were detected for Notch1 and Notch3. Although Notch3 was down-regulated in bile duct cells in all models of regeneration, Notch1 expression was specifically absent in bile duct cells and oval cells in livers treated according to the AAF/PHx protocol.

Cellular Localization of Dlk Protein in Regenerating Liver

In normal or control adult rat liver no immunoreactivity for dlk was detected with antibodies directed against the extracellular (ie, FA1) or intracellular domains of dlk, Figure 2, G and H, respectively. This was in stunning contrast to the immunoreactivity observed in the liver regenerating after treatment according to the AAF/PHx protocol, in which the ductular structures of oval cells reacted strongly with both antibodies. Strikingly, the bile

Figure 2. Immunolocalization with antibodies against PCNA (**A** and **I**), Notch1 (**B** and **J**), Notch2 (**C** and **K**), Notch3 (**D** and **L**), Delta1 (**E** and **M**), Jagged1 (**F** and **N**), and an antibody against the extracellular domain (**G** and **O**) or intracellular domain (**H** and **P**) of *dlk* in serial sections of control liver 5 days after a sham operation (**A–H**) or 9 days after a 70% partial hepatectomy in the AAF/PHx protocol (**I–P**). Serial sections of control liver features a complete portal area while the sections from the regenerating liver feature a portal area with a bile duct and an intricate network of atypical ductular reactions penetrating the hepatic parenchyma.

Table 1. Summary of Immunohistochemical Expression Analysis

Gene/disorder	B.D.C	T.A.D.C	Hepatocytes	Foci of small hepatocytes
<i>Notch 1</i>				
Control/sham	+++	NA	—	NA
PHx 1d	+++	NA	++	NA
AAF/PHx 9d	—	—	++	+
Ret/PHx 5d	++	—	++	++
<i>Notch 2</i>				
Control/sham	++	NA	++	NA
PHx 1d	+	NA	++	NA
AAF/PHx 9d	+	+	+	++
Ret/PHx 5d	+	+	++	++
<i>Notch 3</i>				
Control/sham	+++	NA	++++	NA
PHx 1d	+	NA	++++	NA
AAF/PHx 9d	—	—	+++	—
Ret/PHx 5d	—	—	+++	+++
<i>Delta1</i>				
Control/sham	—	NA	+	NA
PHx 1d	—	NA	++	NA
AAF/PHx 9d	—	—	+ / + + *	—
Ret/PHx 5d	—	—	++	—
<i>Jagged1</i>				
Control/sham	+++	NA	+	NA
PHx 1d	+++	NA	+	NA
AAF/PHx 9d	+++	+++	—	—
Ret/PHx 5d	+++	+++	+	—
<i>Dlk</i>				
Control/sham	—	NA	—	NA
PHx 1d	— / (+) *	NA	—	NA
AAF/PHx 9d	—	++++	—	+++
Ret/PHx 5d	—	++	— / (+) †	—
<i>PCNA</i>				
Control/sham	+	NA	+	NA
PHx 1d	+	NA	++++	NA
AAF/PHx 9d	++	++++	—	+++
Ret/PHx 5d	++	++	++++	++++

*(+) Rare cells found in bile ductules.
†(+) Rare positive hepatocyte.

duct cells, mature hepatocytes, and the endothelium of the hepatic arteries and veins were completely devoid of any reactivity (Figure 2, O and P).

In accordance with results obtained with mouse and human tissues, delta-like protein was clearly expressed in fetal rat liver where it was localized to the cytoplasm and membrane of hepatoblasts (Figure 3A) and this expression was completely abolished in hepatocytes as well as other hepatic cells in the normal adult rat liver (Figure 3B). In the AAF/PHx model of adult liver regeneration, treatment with 2-AAF activates cells in the bile ductules to give rise to a small population of oval cells.⁴² The subsequent partial hepatectomy provides the necessary growth stimulus for transient amplification of oval cells before they differentiate within the hepatic lineage to hepatocytes or bile duct cells.^{3,42} Additionally, in the AAF/PHx protocol of liver regeneration oval cells may differentiate along the intestinal cell lineage giving rise to intestinal-like structures within the hepatic parenchyma. The phenotype of oval cells resembles that of bipotential hepatoblasts in the distinct expression of AFP, which on differentiation into mature hepatocytes or bile duct cells is turned off. Because of this, AFP is to date the most accepted marker for hepatoblasts as well as oval cells in adult rat liver. To investigate if dlk could represent an-

other candidate marker for hepatoblasts and oval cells, we initially performed immunohistochemical localization on a time-course experiment in the AAF/PHx protocol. At day 5 after PHx immunoreactive cells were detected in the portal areas (Figure 3C). The cells were either integrated into well-defined duct structures (Figure 3C, inset) or appeared as small clusters of a few cells at the periphery of the portal tract area. Most cells in the bile ducts, the mature hepatocytes, and all other cell types in the liver did not react. At day 7 after PHx, immunoreactive atypical ductular structures of oval cells with a poorly defined lumen (Figure 3D, inset) had formed in the hepatic parenchyma close to the portal tract (Figure 3D) whereas mature hepatocytes and bile duct cells remained unreactive. At day 9 after PHx, ductular structures of oval cells deeply infiltrated the hepatic parenchyma (Figure 3E). At this time, immunoreactive ductular structures with a poorly defined lumen were closely associated with immunoreactive small hepatocyte-like cells, which were then further associated with immunoreactive small hepatocytes and unreactive mature hepatocytes [Figure 3, E (inset) and F]. The immunoreactive small hepatocyte-like cells were often associated with the newly formed foci of small basophilic hepatocytes (Figure 3I). The apparently progressive differentiation of immu-

Figure 3. Immunolocalization of dlk in fetal liver at day 21 after gestation (A); normal adult rat liver (B); 5 days (C), 7 days (D), and 9 days (E) after a 70% partial hepatectomy in the AAF/PHx protocol, an area featuring atypical ductular reactions and the transition between oval cells and small basophilic hepatocytes (arrows) at day 9 after PHx in the AAF/PHx protocol (F); an area featuring the transition between negative and positive ductular epithelial cells at day 9 after PHx in the AAF/PHx protocol (G, arrows point to positive cells with strong dlk expression and arrowheads to cells with weaker expression); an area featuring intestinal-type structures (arrows) at day 9 after PHx in the AAF/PHx protocol (H); a basophilic focus of small hepatocytes at day 9 after PHx in the AAF/PHx protocol (I); and double immunostaining with antibodies against desmin (brown) and dlk (purple) at day 9 after PHx in the AAF/PHx protocol, identifying the desmin-positive Ito cells (arrows) and the dlk-positive cells in atypical ductular reactions (asterisks) (J).

nonreactive oval cells into hepatocytes was associated with a progression from a strong intracellular and membrane immunoreactivity in oval cells to a membrane-associated reactivity in the small hepatocyte-like progeny resembling that of hepatoblasts in the fetal liver (Figure 3, A and F). A likewise progressive differentiation of oval cells into bile duct cells was implied by the close association of unreactive cells forming well-defined bile ducts

with immunoreactive oval cells forming atypical ductular structures with a poorly defined lumen (Figure 3G). Cells forming intestinal-like structures did not react with the antibody against dlk (Figure 3H). To verify that the dlk immunoreactive cells were indeed oval cells and not stellate (also called fat storing or Ito) cells we performed a double immunostaining with antibodies against desmin and dlk. The desmin immunoreactive stellate cells clearly separated from the dlk immunoreactive atypical ductular structures of oval cells (Figure 3J).

Finally, to verify that the expression of dlk was indeed associated with the phenotype of transit-amplifying ductular (oval) cells we studied the cellular protein localization in the adult rat liver responding to 1) a partial hepatectomy by replication of existing mature hepatocytes and bile duct cells, 2) a bile duct ligation by replication of mature bile duct cells, and 3) the retrorsine/PHx protocol by proliferation of small hepatocyte-like progenitor cells. In the PHx model, hepatic cells including hepatocytes and bile duct cells showed nuclear immunoreactivity for PCNA 24 hours after surgery (Figure 4A). As in control liver, no immunoreactivity for dlk could be detected (Figure 4B). Similarly, cells showing nuclear immunoreactivity for PCNA could be detected in the bile ducts 5 days after a ligation of the common bile duct, but no immunoreactivity against dlk was found (Figure 4, C and D). However, in the retrorsine/PHx protocol small clusters of dlk immunoreactive cells could be detected at the periphery of the portal tracts 5 days after PHx (Figure 4F). The immunoreactive cells corresponded to the oval cell compartment previously described as activated in this model of regeneration.⁹ The small hepatocyte-like progenitor cells, mature hepatocytes, bile duct cells, and hepatic endothelial cells were all unreactive.

Sorting of Dlk Immunoreactive Fetal Hepatoblasts and Transit-Amplifying Ductular (Oval) Cells

Our antibody against the rFA1 variant encoded by *DLK1* recognizes the extracellular domain of the transmembrane dlk protein. Therefore, we speculated that this antibody could be used to sort fetal rat hepatoblasts as well as adult transit-amplifying ductular (oval) cells from suspensions of isolated liver cells. For this purpose we developed a novel, very simple, and quick method for the enrichment using a functional antibody surface where the antibody is cross-linked to the plastic surface of a Petri dish with an anthraquinone conjugate. On this surface hepatoblasts isolated by a collagenase digestion from fetal rat liver 21 days after gestation were able to attach (Figure 5aA) and proliferate for at least 5 days in culture (Figure 5aB). Only a very limited number of cells adhered when the plastic surface was simply coated with the anthraquinone conjugate (Figure 5aA, inset). A semi-quantitative RT-PCR for AFP showed a low expression in the nonsorted cell suspension, an undetectable expression in the nonadhering cells, and a high expression in the cell populations adhering to the surface coated with the antibody against dlk confirming that the attached

Figure 4. Identification of dlk-expressing cells within the liver cell lineage. Expression of PCNA (**A**) and Dlk (**B**) 1 day after a 70% hepatectomy; PCNA (**C**) and dlk (**D**) 5 days after ligation of the common bile duct; PCNA (**E**) and dlk (**F**) 5 days after a 70% hepatectomy in the Ret/PHx model; PCNA (**G**) and dlk (**H**) 5 days after a 70% hepatectomy in the AAF/PHx protocol. **Short arrows** point to atypical ductular reactions composed of transit-amplifying oval cells; **long arrows** point to a focus of small hepatocyte-like cells. The sections stained for PCNA and dlk in each experimental model represent serial sections of a portal area.

cells were indeed hepatoblasts. The hepatoblasts still expressed high levels of AFP after 5 days in culture (Figure 5b, embryonic liver day 21). Likewise, when non-parenchymal cell populations were isolated by collagenase/pronase perfusion from an adult rat liver treated according to the AAF/PHx protocol at day 10 after PHx a large number of small cells were capable of preferential adhesion to the surface coated with the antibody against dlk (Figure 5aC). The adhering cells expressed higher levels of AFP than the nonsorted and nonadhering cell populations (Figure 5b, AAF/PHx day 10) and were, therefore, of an oval cell phenotype. Finally, when non-parenchymal liver cell populations from normal rats were isolated by collagenase/pronase perfusion no cell adherence to the surface coated with the antibody against dlk over background was found, in accordance with the lack of dlk expression in normal liver (Figure 5aD). Neither the nonsorted nor the nonadhering cells isolated from normal liver expressed any significant levels of AFP (Figure 5b, control).

Discussion

The well-documented involvement of the Notch pathway in cell fate determination during development prompted us to examine the expression patterns of the Notch1,

Notch2, and Notch3 receptors, their ligands Jagged1 and Delta1, and dlk in a number of different models of liver injury and regeneration, each characterized by their ability to call forth a cellular response at different levels in the hepatic lineage. Our studies demonstrate differing patterns of gene expression in the various models suggesting that these genes play distinct roles in determination of cell fate within the hepatic lineage.

With respect to the steady-state mRNA levels of Notch receptors and their ligands Jagged1 and Delta1, transcripts were detected for all genes in normal liver and different models of regeneration with the most pronounced changes found in liver regenerating by recruitment of transit-amplifying ductular (oval) cells (AAF/PHx protocol). In normal adult rat liver, Notch1, Notch2, Notch3, and Jagged1 expression was detected by immunohistochemistry on bile duct cells whereas Notch2, Notch3, and Delta1 was found on normal adult rat hepatocytes. In contrast, ductular reactions composed of oval cells as well as newly formed bile ducts in the regenerating rat liver expressed Jagged1 and Notch2 but did not express Notch1, Notch3, or Delta1 whereas mature hepatocytes expressed Notch1, Notch2, Notch3, and Delta1. The finding of Jagged1 and Notch2 expression in bile duct cells and structures of transit-amplifying ductular cells is in agreement with recent studies showing a

a

b

Figure 5. a: Isolation of fetal hepatoblasts and adult transit-amplifying oval cells based on *dlk* expression. Cells were selected on plastic surface covalently linked to an antibody against the extracellular domain of rat *dlk*. Fetal hepatoblasts isolated at day 21 after gestation (**A**, inset shows cell binding to the negative control without antibody linking) and after 5 days in culture (**B**). **C:** Adult transit-amplifying oval cells isolated at day 10 after the 70% partial hepatectomy in the AAF/PHx protocol (inset shows negative control). **D:** Adult nonparenchymal cells isolated from a normal adult rat liver (inset shows negative control). **b:** Semiquantitative RT-PCR of AFP expression on the cell populations isolated in **a**. Twenty ng of total RNA was reverse-transcribed and the cDNA amplified by PCR. The PCR products were visualized by agarose gel electrophoresis and staining with SYBR Green II.

conclusive role of these two gene products in bile duct epithelial cell differentiation and morphogenesis in the mouse.⁴⁵ The absence of Notch1 and Notch3 in oval cells and bile duct cells in the AAF/PHx protocol as compared to mature bile duct cells in normal liver, suggests that in rat liver Notch1 and Notch3 may be involved in the final differentiation and maintenance of the mature bile duct cell. Because Notch3 is thought to function as an inhibitor of Notch signaling,⁴⁶ co-expression of Notch3 with Notch1, Notch2, and Jagged1 in the bile duct cells and of Notch3 with Notch2 and Delta1 in hepatocytes suggests that the Notch signaling pathway could be subject to both positive and negative control in normal adult

rat liver. It is possible that Notch1 and Notch2 receptors are involved in the final cell specification while repression of Notch signaling by the Notch3 repressor is required to maintain these mature cells in a differentiated and non-proliferative state. Along these lines, the specific absence of Notch1 and Notch3 expression in oval cells as compared to surrounding hepatocytes may confer a regenerative advantage to these cell populations. In the AAF/PHx protocol as compared to other models of liver regeneration used in the present study, impairment of hepatocyte proliferation is thought to be particularly important to initiate activation and transient expansion of the endogenous progenitor cell compartment. However, we did not detect any significant changes in Notch receptors and ligands with respect to the hepatocyte populations when the AAF/PHx protocol was compared to the PHx and Ret/PHx protocols of regeneration. In the latter two protocols hepatocyte proliferation is an important part of the regenerative response indicating that Notch signaling is not regulated at the gene expression level in hepatocytes responding to a regenerative stimulus. Overall, our expression studies on genes involved in Notch signaling are in good agreement with studies performed by Nijjar and colleagues^{39,40} in normal and diseased human liver tissue and support their conclusions of the Notch signaling pathway being important for normal bile duct morphogenesis and differentiation.

In the present study, the most striking changes in gene expression were observed for *dlk*. In accordance with studies in mouse and human tissues,^{34,41} rat *dlk* is highly expressed during embryonic development in hepatoblasts but is down-regulated on their differentiation into ductal plate cells and eventually biliary epithelial cells (HC Bisgaard, unpublished observations). On birth, hepatoblasts differentiate into hepatocytes and down-regulate *dlk*. After liver injury, *dlk* expression reoccurs first in a few cells scattered in the existing duct and ductular structures and later all cells in the atypical ductular structures as well as their immediate hepatocyte-like progeny express the protein. However, cells in well-defined bile ducts are consistently negative. Substantial evidence has accumulated that *dlk*, a transmembrane protein belonging to a family of EGF-like repeat-containing proteins that include Notch/Delta/Serrate, is involved in cell-fate determination. *Dlk1* was originally cloned in a cDNA library screen for genes differentially expressed during adipocyte differentiation using the mouse preadipocyte cell line 3T3-L1.³¹ On differentiation of the preadipocytes, the abundantly expressed *dlk* transcript was down-regulated and could not be detected in mature adipocytes. Forced expression of *dlk* in 3T3-L1 cells was reported to inhibit adipogenesis, whereas suppression of *dlk* promoted the process and accordingly, *dlk* was suggested to play an important role in the maintenance of the preadipose state. In support of this hypothesis, recent studies on *dlk1*-null mice demonstrated that lack of *dlk* expression was associated with obesity⁴⁷ while transgenic mice expressing the full ectodomain of *dlk* (dimeric form) exhibited inhibition of adipogenesis and impairment of adipocyte function leading to development of metabolic abnormalities.⁴⁸ Apart from its func-

tion in adipocyte differentiation, dlk has been shown to participate in cell-to-cell interactions occurring in the hematopoietic environment of the bone marrow through regulation of stromal cell signals involved in both self-renewal and differentiation of hematopoietic stem cells into highly proliferative myeloid-erythroid precursors.^{49,50} A recent report has also described a role for dlk in thymocyte development in which a dimeric form of dlk was shown to increase thymocyte cellularity in an HES-dependent manner even though dlk is devoid of the DSL motif generally thought to be necessary for the Delta-Notch signaling though HES-1, a member of the HES family of basic helix-loop-helix transcription factors.⁵¹ Furthermore, dlk has been suggested to participate in the zonal differentiation of the adrenal cortex.⁵² From these observations, a model for dlk function based on the hypothesis of Laborda,³⁸ and analogous to the cell specification hypothesis for Notch and Delta, can be put forward to explain the role of dlk expression in adult liver regeneration. According to this model, the early expression of dlk in an activated putative endogenous stem cell located in the canal of Hering may suppress the differentiation of its progeny and permit their transient expansion in atypical ductular reactions.

In the present study, expression of dlk was closely associated with the transit-amplifying populations of ductular (oval) cells expressing high levels of PCNA protein, a nuclear marker of cell proliferation. Dlk has, apart from its function in differentiation, been implicated as a growth factor. Like other members of the EGF-like protein family that exert their effects through protein-protein interactions, dlk may function as a soluble or transmembrane protein and play important roles in cellular growth and proliferation. One well-characterized subclass includes EGF and transforming growth factor- α , molecules that have been implicated as players in atypical ductular reactions in adult liver.^{53,54} These proteins arise by ectodomain processing and release from transmembrane precursors and exert their action on binding to the EGF receptor. Such an ectodomain processing also applies to dlk.⁵⁵ It is unlikely that dlk binds to the EGF receptor because none of the six EGF-like repeats contains the same spacing of cysteine and other amino acids crucial for binding to the EGF receptor. In pancreas, expression of dlk is high during increased mitotic activity of β cells, ie, in the perinatal period, during pregnancy, and by exposure to growth factors such as growth hormone and prolactin.⁵⁶ However, in a recent study with RINm5F rat insulinoma cells stably expressing human dlk it was observed that dlk decreased both their proliferation rate and their insulin content, supporting the idea that dlk may act as a dedifferentiation factor without being a growth factor per se.⁵⁷ This has led to the hypothesis that up-regulation of dlk may lead to suppression of certain differentiated functions that may facilitate responsiveness to mitogenic stimuli in adjacent cells thereby maintaining proliferation of a population of undifferentiated stem/progenitor cells. Such a mechanism has also been suggested for hematopoietic stem cells, in which stromal cells expressing dlk were found to support self-renewal of the stem cells.⁵⁰

The usage of intrahepatic oval cell progenitors for transplantation has wide therapeutic implications. However, as atypical ductular reactions in rodent as well as human liver diseases are composed of cells with heterogeneous phenotypes studies on the repopulation potential of oval cells have been hampered by the lack of suitable markers for their isolation as homogenous cell populations. In fact in a number of studies, oval cell markers have been identified that are also expressed in the bile duct cells (ie, CD34, c-kit, Ov6, γ -glutamyl transpeptidase) or in hematopoietic stem cells (CD34, c-kit, Thy-1, Sca-1)^{58–61} limiting the usability of these markers for isolation of a homogenous population of oval cells. Our observation of rat dlk expression in hepatoblasts during embryonic development, its down-regulation in hepatocytes on birth, and reoccurrence first in a limited number of ductular cells and subsequently in the transiently amplifying populations of oval cells and their immediate hepatocyte-like progeny is intriguing. We have previously observed a similar pattern for expression of AFP during the early stages of oval cell proliferation⁶² in which a subset of cells in the biliary ductules expressed high levels of transcript. We now hypothesize that dlk and AFP are indeed expressed on activation of an endogenous stem cell compartment and represent unique markers for the adult transit-amplifying ductular (oval) cells. Although AFP is a secreted protein, dlk exists as a transmembrane protein where the extracellular domain can be cleaved resulting in a soluble ectodomain. The fact that dlk is a transmembrane protein led us to the isolation and apparent enrichment of AFP protein-positive hepatoblasts from fetal liver and oval cells from adult regenerating liver. While this work was in progress, Tanimizu and colleagues⁴¹ isolated transplantable hepatoblasts from mouse embryonic liver based on their expression of dlk. Together our results indicate that dlk is a useful marker to enrich the highly proliferative hepatoblasts from fetal liver as well as the transit-amplifying populations of ductular (oval) cells from the adult regenerating liver.

In recent years, the work in our laboratory has been focused on identifying novel molecular pathways involved in activation and differentiation of transit-amplifying ductular (oval) cells.⁶³ These studies have provided us with a wealth of information regarding novel proteins that may be used to separate transit-amplifying ductular (oval) cells on a molecular basis from other populations of nonparenchymal cells that are co-isolated by protease digestion of liver tissue. Together with the data provided by the present studies on dlk we now have tools to further characterize the transit-amplifying ductular (oval) cells and their therapeutic potential in adult liver regeneration.

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