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BASIC AND TRANSLATIONAL—LIVER

Hedgehog Controls Hepatic Stellate Cell Fate by Regulating Metabolism

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BACKGROUND & AIMS: The pathogenesis of cirrhosis, a disabling outcome of defective liver repair, involves deregulated accumulation of myofibroblasts derived from quiescent hepatic stellate cells (HSCs), but the mechanisms that control transdifferentiation of HSCs are poorly understood. We investigated whether the Hedgehog (Hh) pathway controls the fate of HSCs by regulating metabolism. **METHODS:** Microarray, quantitative polymerase chain reaction, and immunoblot analyses were used to identify metabolic genes that were differentially expressed in quiescent vs myofibroblast HSCs. Glycolysis and lactate production were disrupted in HSCs to determine if metabolism influenced transdifferentiation. Hh signaling and hypoxia-inducible factor 1α (HIF1α) activity were altered to identify factors that alter glycolytic activity. Changes in expression of genes that regulate glycolysis were quantified and localized in biopsy samples from patients with cirrhosis and liver samples from mice following administration of CCl_4 or bile duct ligation. Mice were given systemic inhibitors of Hh to determine if they affect glycolytic activity of the hepatic stroma; Hh signaling was also conditionally disrupted in myofibroblasts to determine the effects of glycolytic activity. **RESULTS:** Transdifferentiation of cultured, quiescent HSCs into myofibroblasts induced glycolysis and caused lactate accumulation. Increased expression of genes that regulate glycolysis required Hh signaling and involved induction of HIF1α. Inhibitors of Hh signaling, HIF1α, glycolysis, or lactate accumulation converted myofibroblasts to quiescent HSCs. In diseased livers of animals and patients, numbers of glycolytic stromal cells were associated with the severity of fibrosis. Conditional disruption of Hh signaling in myofibroblasts reduced numbers of glycolytic myofibroblasts and liver fibrosis in mice; similar effects were observed following administration of pharmacologic inhibitors of Hh. **CONCLUSIONS: Hedgehog signaling controls the fate of HSCs by regulating metabolism. These findings might be applied to diagnosis and treatment of patients with cirrhosis.**

Keywords: Mouse Model; Liver Disease; Signal Transduction; Gene Regulation.

Cirrhosis occurs when excessive numbers of myofibroblasts (MFs) accumulate in injured livers. Many of these MFs are derived from quiescent hepatic stellate cells (Q-HSCs) via a transdifferentiation-like process whereby the cells shift from a quiescent lipogenic state to become proliferative and fibrogenic.[1] Researchers have identified a number of injury-related factors that promote hepatic stellate cell (HSC) transdifferentiation and MF growth. Conversely, MF populations gradually involute, and fibrosis tends to regress, when injury is cured.[2] The global power of the microenvironment in dictating the fate of HSCs is further supported by evidence that targeted efforts to constrain MF accumulation during active liver injury have failed. These observations suggest that redundant injury-initiated mechanisms activate the molecules that ultimately control the fate of HSCs and justify work to identify those master regulators.

There is emerging evidence that Hedgehog (Hh), a master developmental regulator,[3,4] becomes reactivated during adult wound healing.[5] The Hh pathway is activated when Hh ligands bind to their receptor Patched on the surface of Hh-responsive cells. This relieves the inhibitory actions of Patched on Smoothened (SMO), the signaling competent Hh coreceptor. SMO then translocates to the cilium, where it promotes the nuclear localization of the transcription factors glioblastoma (GLI)1, GLI2, and GLI3, which control the expression of Hh target genes. Most of the biological effects of the Hh pathway are believed to result from transcriptional regulation of GLI target genes, whose products influence stem cell renewal and lineage decisions as well as cell cycle progression and cell migration.[6–8] Although the exact role of Hh in adult tissue repair remains somewhat obscure, the pathway

Abbreviations used in this paper: α-SMA, α–smooth muscle actin; BDL, bile duct ligation; 2DG, 2-deoxy-glucose; DTG, double-transgenic; FBP, fructose bisphosphatase; GFP, green fluorescent protein; GLI, glioblastoma; Hh, Hedgehog; HIF1α, hypoxia inducible factor 1α; HSC, hepatic stellate cell; MDR, multidrug resistant; MF, myofibroblast; PH, partial hepatectomy; PK, pyruvate kinase; Q-HSC, quiescent hepatic stellate cell; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SMO, smoothened.

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seems to control critical cell fate decisions that are required for reconstruction of healthy tissue because cancers and fibrosis typically result when Hh signaling becomes deregulated.[6,9,10] Inhibiting Hh signaling also blocks adipogenesis,[11] suggesting a role for the Hh pathway in metabolism. However, it is not known if the metabolic effects of Hh influence its other actions or vice versa.

Like several of the key cell types involved in liver repair, HSCs are Hh responsive.[6,12] Hh pathway activation promotes transition of Q-HSCs into MF-HSCs, and pathway inhibition drives MF-HSCs to revert back to a quiescent phenotype.[13] We hypothesized that Hh signaling might direct the fate of HSCs by regulating their metabolism because Q-HSCs are adipocyte-like[2] and loss of lipid is a hallmark of Q-HSC transition to MF.[1,14,15]

Materials and Methods

Full methods are available in Supplementary Materials and Methods.

Primary HSC Studies

Primary HSCs were isolated from adult rats, wild-type C57BL6 mice, *Smo*^{tm2Amc}/J (SMO-LoxP) mice,[16] or mtGFP transgenic mice[17] and culture activated for up to 7 days in Dulbecco's modified Eagle medium containing either 4500 or 1000 mg/L glucose.[18,19] Four-day cultures were treated with inhibitors of glycolysis (2-deoxy-glucose [2DG]; Sigma-Aldrich, St Louis, MO), lactate dehydrogenase (FX11),[20] SMO (GDC-0449; Selleck Chemicals, Houston, TX),[21] or hypoxia-inducible factor (HIF)1α (acriflavine; Sigma-Aldrich)[22] and analyzed on day 7. In HSCs from SMO-LoxP mice, Cre-recombinase adenoviral vectors were used to delete the floxed SMO allele. Results were compared with HSCs treated with green fluorescent protein (GFP) adenoviral vectors.

Animal Studies

C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used to model liver injury. All studies were approved by the Duke University Institutional Animal Care and Use Committee as set forth in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Acute liver injury models. *CCl_4.* Wild-type male mice were injected intraperitoneally with olive oil (n = 2) or CCl_4 (CCl_4/olive oil = 1:20; n = 6) and killed on day 2, 4, or 7.

Partial hepatectomy. Adult male mice were subjected to partial hepatectomy (PH) and treatment with cyclopamine as described.[23] Livers were harvested at the time of PH (n = 6) or after 48 hours (n = 6).

Chronic liver injury models. *Bile duct ligation.* Wild-type male mice underwent bile duct ligation (BDL; n = 5) or sham surgery (n = 4) and then were killed after 14 days. SMO-LoxP mice[16] (Jackson Laboratories) were crossed with α–smooth muscle actin (α-SMA)-Cre-ERT2 transgenic mice to generate double-transgenic (DTG) mice in which treatment with tamoxifen induces conditional deletion of SMO in α-SMA–positive cells. Adult (8–12 weeks) mice were subjected to BDL or sham surgery (n = 8 mice/group); treated with vehicle or tamoxifen on days 4, 6, 8, and 10 after BDL; and killed 14 days after BDL.

Methionine-choline–deficient diet. Wild-type male mice were fed control (n = 2) or methionine-choline–deficient diets (MP Biomedicals, Solon, OH; n = 3) for 8 weeks.

MDR2$^{-/-}$ *mice.* Aged (52–62 week old) MDR2$^{-/-}$ mice were treated with vehicle (n = 10) or GDC-0449 (n = 10) for 9 days.[24]

Human Studies

Anonymized healthy and diseased human livers were examined for expression of glycolysis markers as per institutional review board–approved protocols.

Statistical Analysis

Results are expressed as mean ± SEM. Analyses were performed using Student *t* test. $P < .05$ was considered significant.

Results

Metabolism Is Reprogrammed During HSC Transdifferentiation

We performed microarrays to screen HSCs for transition-associated changes in metabolism. To capture early, as well as late, events in the transdifferentiation process, gene expression was compared in freshly isolated primary HSCs and HSCs after 7 days in culture. This approach differed from earlier studies that examined HSCs cultured for 1 day or more.[25] We found that a significant number of the genes that are differentially expressed in Q-HSCs vs MF-HSCs are involved in metabolism (Supplementary Tables 1 and 2). This point had not been emphasized previously and suggests that alterations in HSC metabolism occur rapidly during their transdifferentiation. The gene expression profile of MF-HSCs also resembled that of highly proliferative cells.[26] Because proliferative cells are often glycolytic,[26,27] we compared glycolytic activity in Q-HSCs and MF-HSCs. MF-HSCs showed significantly increased expression of glycolytic enzymes (Figure 1*A–C* and Supplementary Figure 1*A*) and glucose and lactate transporters (Figure 1*D*), with coincident down-regulation of genes involved in gluconeogenesis (Figure 1*E*). Notably, the most striking differences in many of these transcripts occurred within the initial 48 hours of culture. Hence, the metabolic shifts would have been overlooked if gene expression had been evaluated only at later time points. For example, messenger RNA (mRNA) levels of the glucose transporter Glut1 and 2 key glycolytic enzymes, hexokinase 2 and pyruvate kinase (PK)M2,[28] increased 6- to 25-fold within 6–24 hours in culture (Supplementary Figure 2) and remained elevated on culture day 7 (Figure 1*A*). Western blot and immunocytochemistry confirmed that expression of these glycolytic enzymes was rapidly switched on when Q-HSCs were placed into culture and then maintained at high levels (Figure 1*B* and *C*). Conversely, transcripts for phosphoenolpyruvate carboxykinase 1 and fructose bisphosphatase (FBP)1, 2 key gluconeogenic enzymes,[26] plummeted by 90% during the initial 48 hours in culture and remained extremely low on culture day 7 (Figure 1*E*).

As previously described,[1] expression of genes involved in lipid synthesis, oxidation, and uptake decreased by 50%–90% during early HSC culture and remained low thereafter (Supplementary Figure 1*C*). Unlike the early