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REVIEW

Contribution of human hematopoietic stem cells to liver repair

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Abstract Immune-deficient mouse models of liver damage allow examination of human stem cell migration to sites of damage and subsequent contribution to repair and survival. In our studies, in the absence of a selective advantage, transplanted human stem cells from adult sources did not robustly *become* hepatocytes, although some level of fusion or hepatic differentiation was documented. However, injected stem cells did home to the injured liver tissue and release paracrine factors that hastened endogenous repair and enhanced survival. There were significantly higher levels of survival in mice with a toxic liver insult that had been transplanted with human stem cells but not in those transplanted with committed progenitors. Transplantation of autologous adult stem cells without conditioning is a relatively safe therapy. Adult stem cells are known to secrete bioactive factors that suppress the local immune system, inhibit fibrosis (scar formation) and apoptosis, enhance angiogenesis, and stimulate recruitment, retention, mitosis, and differentiation of tissue-residing stem cells. These paracrine effects are distinct from the direct differentiation of stem cells to repair tissue. In patients at high risk while waiting for a liver transplant, autologous stem cell therapy could be considered, as it could delay the decline in liver function.

Keywords Hematopoietic stem cell · Hepatocytes · Cell fusion · Aldehyde dehydrogenase · Liver damage · Regenerative medicine

Abbreviations

AAT	α-1-antitrypsin
ALB	albumin
ALDH	aldehyde dehydrogenase
$ALDH^{hi}$	high activity of ALDH
$ALDH^{lo}$	low activity of ALDH
BMSC	bone marrow stem cell
CCl_4	carbon tetrachloride
DAPI	4,6-diaminidino-2-phenylindole
FACS	fluorescence-activated cell sorting
FAH	fumarylacetoacetate hydrolase
FISH	fluorescent in situ hybridization
GUSB	beta-glucuronidase
hCBSC	human cord blood stem cells
HNF1	hepatocyte nuclear factor 1
Lin^-	lineage-depleted
MPS VII	mucopolysaccharidosis type VII
NOD/SCID	nonobese diabetic/severe combined immunodeficient

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Tissue-dependent fusion and stem-cell-induced repair of nonhematopoietic tissues including the liver

"Plasticity" refers to the putative ability of adult stem cells to acquire mature cell phenotypes distinct from their tissue of origin. Reports had initially described that transplanted bone marrow (BM)-derived stem cells could generate cells with unexpected phenotypes, including cardiac and skeletal muscle, brain, endothelial, endocrine, and epithelial line-

ages, in addition to liver (reviewed in [1, 2]). These phenomena occurred at a low frequency in vivo [3] and required tissue damage for stem cell recruitment and selective survival advantage for propagation [4]. Several reports then failed to reproduce original findings [5–7] but other studies demonstrated that transplanted BM cells engrafted nonhematopoietic tissues and stimulated improved function of the damaged organs in vivo [4, 8–10]. The biological mechanisms responsible for "stem cell plasticity" are still debated, especially in the liver. Recent studies have shown stem cell fusion with mature tissue-specific cells [11–13]. Fusion of transplanted BM-derived cells with damaged cells has been implicated in the production of skeletal muscle [14], neural [15], cardiac [6], and hepatic cells [12, 13, 16]. Cre-recombinase-mediated activation of reporter genes has been used to detect cell fusion events after bone marrow transplantation in vivo. Alvarez-Dolado et al. used this system to demonstrate donor bone-marrow-derived cell fusion with mature recipient hepatocytes, cardiomyocytes, and Purkinje cells [15]. Two other groups have used similar Cre-loxP strategies to show the absence of cell fusion [17, 18]. Ianus et al. [17] used Y chromosome fluorescent in situ hybridization (FISH), in combination with an insulin-promoter-driven Cre/Lox system, to show the production of BM-derived insulin-producing cells without Cre-mediated activation of enhanced green fluorescence protein through cell fusion. We have shown that, although relatively rare, cell fusion does exist in vivo between transplanted human stem cells and recipient immune-deficient mouse hepatocytes, resulting in human albumin-expressing cells [19]. However, Harris et al. identified epithelial cells in the lung, skin, and liver that develop from BM-derived cells through a mechanism other than cell fusion [18]. Cell fusion may be a cell-type-specific phenomenon that is more common in organs that normally contain cells with different ploidies such as liver and muscle and can be induced by acute physiologic injury. In addition, it seems probable that alternate mechanisms, such as the induction of angiogenic processes [20] and stem-cell-mediated enhancement of cellular proliferation [10], are involved in the cumulative benefit of stem-cell-mediated repair of nonhematopoietic organs.

Liver transplantation remains the only therapeutic option for many acute and chronic end-stage liver diseases. However, this approach is limited by a serious shortage of donor organs required for transplantation. A number of studies have suggested that stem cells from bone marrow could generate hepatocytes (reviewed in [21]). The most successful liver repopulation with bone-marrow-derived cells was observed in mice with fumarylacetoacetate hydrolase (FAH−/−) deficiency resulting in functional correction of the liver disease [4, 12, 13]. In this excellent model, there is a strong selection for corrected donor hepatocytes that express the functional enzyme, in contrast to other liver damage models where the endogenous recipient cells can also contribute to the repair. In the xenotransplantation models with human cells injected into mice, the contribution from the donor human cells to the damaged liver can be easily observed by human-specific markers or centromeric chromosome analysis by FISH that discriminate between murine and human chromosomes (Fig. 1) [22, 23].

Isolation of hematopoietic stem cells for liver repair using phenotypic vs. functional markers

Methods to identify the most primitive hematopoietic stem cells (HSC) are constantly sought. The CD34 protein is frequently used as a marker for positive selection of human hematopoietic stem and progenitor cells. However, Goodell et al. [24, 25] characterized murine Hoechst dye-excluding side population cells that lacked CD34 expression and had reconstitution capacity. Bhatia et al. [26] demonstrated a low level of engraftment activity in human CD34- cells. We showed that highly purified human CD34+ cells generated CD34− cells in immune-deficient mice after 1 year and that the reisolated human CD34− cells retained the capacity to regenerate CD34+ cells upon secondary transplantation. We determined that CD34 expression at the cell surface and messenger RNA (mRNA) level can be turned on and off, relating to the quiescence of the human hematopoietic stem

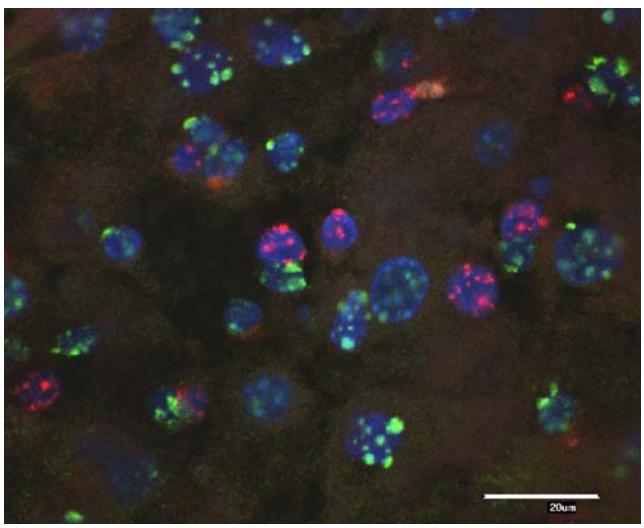

Fig. 1 FISH analysis for human and mouse centromeres in liver sections. Liver sections from a mouse that had been transplanted with $ALDH^{hi}Lin^{-}$ cells after CCL4-mediated liver injury were probed with both human (*red dots* in nuclei) and mouse (*green dots* in nuclei) centromeric probes. The analysis shows that the liver was heavily engrafted with human stem cells and that the majority of the human cells had not undergone fusion with the damaged murine hepatocytes

cell population in vivo [27]. Human CD34+/CD38− cells engraft in beige/nude/xid immune-deficient mice and their progeny can be recovered from the murine bone marrow for as long as 18 months posttransplantation [28, 29]. When reisolated from the marrow of beige/nude/xid mice, the human cells were absolutely devoid of CD34 expression. No CD34 mRNA was detected in the human CD45+ cells, reisolated from the bnx bone marrow. The human CD45+/CD34− cells generated multilineage reconstitution in secondary nonobese diabetic/severe combined immunodeficient (NOD/SCID) recipients. CD34+ cells were recovered from the secondary recipients, demonstrating that CD34 expression is reversible: CD45+/CD34+/CD38− cells can generate CD45+/CD34− cells, which can then generate CD45+/CD34+ cells. Our data agree with reports from Makio Ogawa's group [30], which indicate that CD34 expression may be an activation-dependent event. Hess and Bhatia had independently found that after coculture and transplantation into immune-deficient mice, male CD34-/Lin- cells generated CD34+/CD38− cells, which, upon recovery from the mice, demonstrated clonogenic progenitor function into multiple lineages [31]. These data demonstrated that expression of CD34 is reversible on human multilineage engrafting stem/progenitor cells [27, 32]. This calls for different purification strategies, based on conserved stem cell function as well as phenotype, to avoid "missing" the most primitive or multipotent cells within the bone marrow.

Since we and others have shown that human hematopoietic cell phenotype can vary with activation state [27, 30, 31, 33], we therefore sought a functional rather than phenotypic method for identifying human stem cells with regenerative potential. The use of metabolic markers such as rhodamine and Hoechst 33342 dye efflux separates cells based on high expression of membrane pumps encoded by the multiple drug resistance genes, a characteristic believed to be associated with very immature stem/progenitor cells [24, 25, 34, 35]. Another very interesting metabolic marker indicative of early immature cells is the enzyme aldehyde dehydrogenase (ALDH), shown by our group and others to correlate with high stem cell activity in vivo [36–38]. In our studies, progenitor function and reconstituting ability were exclusive to the ALDH high and lineage-depleted fraction ($ALDH^{hi}Lin^{-}$), as opposed to the $ALDH^{lo}Lin^{-}$ population [38]. We also examined the combination of ALDH activity with markers such as CD133, the human homolog of prominin 5-transmembrane glycoproteins, which is also a prominent HSC marker [38–43]. Fractionation of human HSC based on high ALDH activity, with and without other markers such as CD133, provided a rigorous selection of purified stem and progenitor cells [38, 44]. We have subsequently shown that $ALDH^{hi}Lin^{-}$ cells represent a unique mixed stem cell population with hematopoietic, mesenchymal, and endothelial progenitor function [45] and have examined the function of this population in tissue repair, including the liver, as described further below.

Role of hepatocyte growth factor in stem cell survival, recruitment, and tissue repair

Hepatocyte growth factor (HGF)/scatter factor is a mesenchymal-derived heparin-binding glycoprotein that has effects on the survival, mitosis, and migration of many cell types. HGF induces gradient-directed migration (chemotaxis) and invasion through extracellular matrix proteins. It induces a program of activities in migrating cells that are required for invasion into tissues [46–48]. The mature molecule is an αB heterodimer that is cleaved from a single polypeptide precursor. When expressed from vectors in mammalian cells, the precursor protein is appropriately cleaved to form the active heterodimer [49, 50]. The receptor for HGF is the transmembrane tyrosine kinase c-Met [51]. Both HGF activation and c-Met expression are inducible by hypoxia. In addition to its established role in stimulation of hepatocyte survival, migration, and division, HGF has been found to play a role in hematopoiesis. The HGF protein is produced by marrow stromal cells, which suggests that it may have a role in the maintenance of HSC. c-Met is expressed on hematopoietic progenitors that respond to HGF [52–60] as well as cells that are capable of regenerating liver and muscle, in its better known roles (reviewed in [61]). c-Met is expressed on immature human marrow-derived hematopoietic stem/progenitor cells with the phenotype CD34+CD33− and CD34+CD38− but not on more mature progenitors with the phenotype CD34+CD33+ and CD34+CD38+ [59].

HGF also plays an important role in tissue repair. During regeneration, expression of HGF from the damaged tissue is upregulated and later downregulated in controlled patterns [62, 63]. The role of HGF in repair has been well documented in the liver. Kupffer cells in the liver upregulate HGF mRNA following liver injury, and expression of HGF by endothelial cells in local capillary beds is also induced [64, 65]. HGF is also upregulated by the mouse heart in response to myocardial ischemia, and, after myocardial infarction in humans, HGF secretion was also upregulated from the infarcted region [66, 67]. HGF also stimulates the activation and early division of adult satellite cells in muscle tissue and is present in injured skeletal muscle [68]. HGF levels are systemically elevated in the serum of patients with liver failure, supporting the idea that the liver may be attempting to recruit stem cells to assist with its repair and regeneration. To gain a better understanding of the role of stem cells in liver and muscle repair, we have examined how these cells might be recruited to

damaged tissues. One of the primary signals that the stem cells receive after tissue injury may be the hypoxia-induced local activation of HGF, which could act in a gradient-dependent manner, to induce stem cell migration into the damaged tissue and to contribute to repair and survival of stem/progenitor cells at the site of damage.

Effects of hypoxia at sites of tissue damage on stem cell phenotype and migration

Recent characterization of the BM microenvironment has shown that the most primitive hematopoietic stem cells are distributed along an oxygen gradient. Primitive stem cells reside in the most hypoxic areas of the BM where low O_2 levels enhance the survival and expansion of primitive self-renewing HSC and endothelial progenitors [69, 70]. Differentiating progenitors reside predominantly in the more vascular sinuses. Progenitor cell trafficking is controlled by chemokine-mediated interactions to regulate the development and release of mature cells into the periphery [71]. CXCR4 and c-Met have both been reported to be expressed on bone marrow-derived HSC and we demonstrated that they can both be increased in hypoxic cultures [72]. CXCR4 has recently been reported to be present as a cytosolic protein pool in addition to its surface expression, raising the intriguing question of whether changes in the microenvironment, such as hypoxia or HGF administration, could enhance the delivery of the cytosolic protein to the extracellular surface. c-Met, the receptor for HGF, is rapidly internalized and degraded following ligand binding. There is evidence from our laboratory and others to show that hypoxia enhances not only the expression of c-Met but also its protein stability and signaling to downstream prosurvival factors [72, 73].

Following chemical-induced liver injury, such as in the CCL4 model that we have reported, damage to the proximal capillaries is a major outcome. The immediate microenvironment surrounding these capillaries will thus be hypoxic. This local hypoxia may induce an upregulation in the active levels of local chemoattractants and growth factors such as HGF and stromal-derived factor 1 in the damaged region of tissue. This combination of low oxygen tension and high chemoattractant gradient may promote the recruitment of tissue- repairing stem cells to the damaged liver site. The fact that marrow-derived stem cells can be found at sites of liver injury (Fig. 2) suggests that they can be recruited to the hypoxic region and that they can survive in the damaged microenvironment. This suggests that stem cells might adapt a phenotype that allows survival in a hypoxic environment, perhaps induced by HGF, a known survival factor for multiple stem cell types.

To explore this theory, we set up an immune-deficient mouse model of liver damage and recovery and determined that CCL4 allowed better human cell engraftment and induction of human albumin expression than radiation or allyl alcohol damage. We transplanted CD34+ or CD34+/CD38−/CD7− purified hematopoietic stem cells from human BM and umbilical cord blood (UCB). CD34+/CD38− cells have been previously defined to be highly quiescent [74–77], and CD34+/CD38−/CD7− cells are a subset of this progenitor pool, shown to generate both lymphoid and myeloerythroid progeny [78]. There was no albumin expression in this population prior to transplantation into the mice [79]. Two months after transplantation into noninjured mice, human hematopoietic cells were present in the murine livers, but none expressed human albumin. However, when CCl_4 was administered 1 month after human stem cell transplantation, the human stem cells were induced to begin expressing human albumin [79]. We

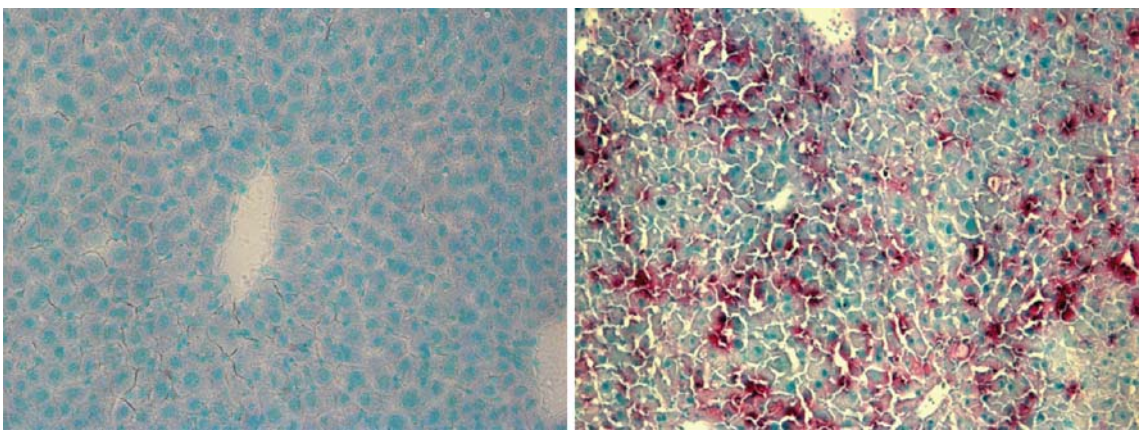

Fig. 2 MPSVII liver. Engraftment of human $ALDH^{hi}Lin^{-}$ cells into mouse livers. Representative frozen section slides from the livers of mice transplanted with human cord blood $ALDH^{lo}Lin^{-}$ (*left*) or $ALDH^{hi}Lin^{-}$ cells (*right*). Liver sections were stained for β-glucuronidase (*red*) and counterstained for methyl green (*green* nuclei)

next tested injection of human HGF into the mice vs. transplantation of HGF-expressing mesenchymal stem cells/marrow stromal cells (MSC), using techniques that we had previously described to produce sustained systemic levels of specific growth factors from the engineered cells [80–82]. HGF production from the engineered MSC accelerated and increased the development of human albumin-expressing cells, which could be detected as early as 5 days after the liver injury in some cases [79]. We did not know at that point whether the hepatocyte-like human albumin-producing cells arose via differentiation or fusion, and determining this was a goal of our subsequent studies.

Tissue localization of transplanted human stem cells in immune-deficient mice

We have studied the in vivo localization of transplanted $ALDH^{hi}Lin^{-}$ cells, as well as adipose and marrow-derived MSC subpopulations, into specific tissues of immune-deficient mice, during steady state vs. chronic and acute injury in tissues including liver. In these studies, we are specifically seeking populations of human cells that cannot only home into the injured murine tissues but those that could also mediate repair in a robust manner. These studies were greatly facilitated by the use of a new mouse model, the NOD/SCID/mucopolysaccharidosis type VII (MPSVII) strain developed by Dr. M. Sands [83]. MPSVII is a lysosomal storage disease caused by a deficiency in B-glucuronidase (GUSB) activity. The MPSVII mutation was backcrossed onto the NOD/SCID strain, and human CD34+ cells engraft in the resultant NOD/SCID/MPSVII strain to levels equivalent to the NOD/SCID parent [83]. Tissue slides prepared from this strain allow rapid visualization of human cells which carry normal levels of GUSB, against the background mouse tissues which are null for the enzyme (Fig. 2). Following the enzymatic reaction, slides can be counterstained with antibodies to a tissue-specific protein marker. The enzymatic stain is quite specific, and, although the released enzyme can be taken up by neighboring cells, it is in a processed form no longer detectable by the histochemical analysis. Thus, the individual transplanted human cells stand out vividly against the background GUSB-null murine tissues (Fig. 2). Most notably, human cells expressing GUSB can be detected without reliance on the expression of cell surface markers. This is important in tissue repair studies in hypoxic environments, where the phenotype of homed stem cells can be dramatically different from that of the transplanted population. The use of the GUSB enzyme staining to detect human cells in NOD/SCID MPSVII mice is proving to be more fast and efficient than FISH, although we and others do still use Y chromosome and centromeric FISH for verification of antibody and enzymatic staining methods (Fig. 1).

Because donor cell surface protein expression can be altered in nonhematopoietic tissues due to cell fusion or other mechanisms, we used the NOD/SCID/MPSVII model to study the tissue distribution of $ALDH^{hi}Lin^{-}$ vs. $ALDH^{lo}Lin^{-}$ cells in liver repair. Injection of the $ALDH^{lo}Lin^{-}$ population, which contains committed progenitors but not stem cells, did not produce significant human cell engraftment in any tissue. In contrast, injection of $2–4\times10^{5}$ $ALDH^{hi}Lin^{-}$ cells into sublethally irradiated (300 cGy) NOD/SCID/MPSVII mice demonstrated high levels of hematopoietic cell engraftment in the BM (70.5±15.1%), spleen (7.0±2.8%), and peripheral blood (17.0±10.7%). Significant human engraftment was also detected, with single cell sensitivity, in nonhematopoietic tissues (liver, pancreas, kidney, lung, heart, skeletal muscle, and brain) of mice transplanted with the $ALDH^{hi}Lin^{-}$ cells. An example of the specificity of locating transplanted human cells in murine tissue sections is shown in Fig. 2. GUSB activity was colocalized with CD45 expression in the BM of mice transplanted with $ALDH^{hi}Lin^{-}$ cells. Histochemical staining confirmed the presence of GUSB+ cells that did not express human CD45 and had hepatocyte morphology [84]. These hepatocyte-like GUSB+ cells were also present after the transplantation of the more purified $ALDH^{hi}CD133+Lin^{-}$ subpopulation, which is greatly enriched for human stem cells [44]. In the pancreas, GUSB+ donor cells were localized in ductal regions and surrounding recipient islets. The $ALDH^{hi}Lin^{-}$ UCB cells demonstrated a previously uncharacterized high level of engraftment in nonhematopoietic organs of NOD/SCID MPSVII mice [84]. We next investigated cellular fusion and nuclear reprogramming as a putative mechanism for the production of nonhematopoietic murine cells expressing donor human cell-derived GUSB and albumin.

Gene transfer from donor to recipient cell can potentially occur through fusion, as elegantly demonstrated by other groups in liver [12, 13, 16, 22] and muscle [85]. We wondered whether cell fusion was playing a major role in the tissue repair that we were observing to be mediated by the different human stem cell populations. We used FISH to assess human cells in the damaged livers of immune-deficient mice, using probes for human ALU repeats [79]. This technique proved to be sensitive enough to detect human albumin-expressing hepatocyte-like cells in the heavily damaged mouse livers, and all human albumin-expressing binucleate cells had human DNA expression in both nuclei [79]. However, this technique did not rule out the possible presence of murine DNA in the same cells, which would be a demonstration of fusion.

To examine the issue of fusion in a more detailed manner, we performed a further series of studies that

examined the contribution of human umbilical-cord-blood-derived stem cells (hCBSC) to liver repair. The frequency of hCBSC-derived hepatocytes varies tremendously between different studies and it is still controversial as to whether hCBSC-derived cells can transdifferentiate into hepatocytes or simply fuse to recipient hepatocytes. We used the GUSB-deficient NOD/SCID/MPSVII mouse model for better identification of engrafted cells. We transplanted lineage-depleted human umbilical-cord-blood-derived cells that had been isolated on the basis of high aldehyde dehydrogenase activity ($ALDH^{hi}Lin^{-}$) into irradiated NOD/SCID/MPSVII mice followed by carbon tetrachloride administration to induce liver damage. We found that the $ALDH^{hi}Lin^{-}$ cells engrafted efficiently into the recipient mouse livers and that they improved recovery of the mice from toxic insult. The percentage of human cells in these livers ranged between 3% and 14.2% using quantitative real-time polymerase chain reaction. Human cells expressing liver-specific α-1-antitrypsin mRNA, albumin, and hepatocyte nuclear factor 1 protein were detected in the recipient livers [86]. Interestingly, human vs. murine centromeric fluorescent in situ hybridization analysis on the liver sections demonstrated that most human cells were *not* fused to murine cells (Fig. 1). However, the majority of the rarer human-originated albumin-expressing cells did also carry mouse genetic material and therefore were the product of cell fusion.

We have previously shown that highly purified CD34+CD38−CD7− human cord blood stem cells with known potent hematopoietic potential could give rise to hepatocyte-like cells in immunodeficient mice [79]. However, the frequency of hepatocyte-like cells derived from hematopoietic cells in mouse models is rare [21]. The mechanisms responsible for these phenomena are also still somewhat controversial. Both direct transdifferentiation [18, 87, 88] of bone marrow and umbilical-cord-blood-derived stem cells to hepatocytes and fusion between BMSC-derived cells and recipient hepatocytes have been reported [12, 13, 15, 22, 89–91]. In our most recent studies, limited numbers (0.05%) of hepatocyte-like cells derived from CBSCs were detected in the recipient livers while significant numbers of donor cells, mostly hematopoietic, were engrafted to the liver following damage. Similar low frequencies of bone-marrow-derived hepatocytes were reported by others [21] and thus represent an obstacle to clinical application.

However, CBSC-derived cells may contribute indirectly to liver regeneration. Hematopoietic cells are capable of producing cytokines, such as interleukin 6, tumor necrosis factor alpha, HGF, and others, to stimulate hepatocyte proliferation [92, 93]. It has also been reported that BMSCs can partially repair CCl_4-induced fibrosis of the mouse liver through the activation of metalloproteinase-9 [94]. In agreement with those data, we observed significantly higher levels of survival in the mice that had been transplanted with human $ALDH^{hi}Lin^{-}$ stem cells in comparison to nontransplanted controls and those transplanted with $ALDH^{lo}Lin^{-}$ cells, which represent committed progenitors. We conclude that human stem cells or their progeny may home to the injured liver and release paracrine factors that hasten tissue repair, while fusion of these cells with hepatocytes occurs rarely and contributes to a lesser extent to liver repair [86].

In summary, our data show that human bone-marrow- and cord-blood-derived stem cells or their progeny can fuse with host hepatocytes and that the fused cells gradually lose human genetic material. Our most recent data demonstrate that human albumin-expressing cells were generated from fusion of human cord-blood-derived cells with the recipient murine hepatocytes. The CD34+ and ALDH high stem cell subsets from both marrow and umbilical cord blood did however have a significant potential to repair liver through paracrine effects, and the human ALDH high stem cells caused a significant increase in survival of immune-deficient mice that had been given a toxic liver insult. Our data suggest that the primary beneficial effects from adult stem cell transplantation to injured liver may not be from direct generation of new hepatocytes but rather through the reparative paracrine effects that the engrafted stem cells exert on the damaged tissue.

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