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The Role of Bone Marrow Derived Cells

in a Model of Hepatic Regeneration

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Maria Mazzeo
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The Role of Bone Marrow Derived Cells in a Model of Hepatic Regeneration

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To examine the relationship between liver injury and the appearance of bone marrow derived hepatic cells we performed sex-mismatched bone marrow transplants in mice, with subsequent liver injury. Co-labeling for a marker of donor bone marrow origin and a marker of liver epithelial phenotype allowed us to identify rare marrow-derived hepatocytes at various time points following liver damage. The number of marrow-derived hepatocytes was low, however, and did not allow us to determine if liver-specific injury upregulated this process from baseline. We conclude that while marrow-derived hepatocytes are found, the low level of occurrence in this study makes it impossible to draw a clear temporal relationship between liver damage, recovery and the appearance of donor-derived cells. In addition, we can not say whether liver-specific damage upregulates this phenomenon.

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A. Introduction:

A.1. Liver disease: impact and clinical treatment

Over 26,000 people in the United States die each year from chronic liver disease and cirrhosis, making it the twelfth leading cause of death in the US (1). Cirrhosis is the consequence of chronic liver disease and is characterized by replacement of hepatic tissue by fibrotic scar tissue and regenerative nodules, leading to progressive loss of liver function. Besides the endpoint of death, liver disease and cirrhosis are very costly in terms of human suffering, hospital expense, and lost productivity (1).

At present, the major therapeutic option available for patients suffering from end-stage liver disease is orthotopic liver allograft. Shortage of donor organs, subsequent requirement for lifelong immunosuppression, and the potential for immunologic rejection in the recipient are major limitations of transplantation. Hepatocyte transplantation, a less invasive alternative, is an application hindered by difficulties with obtaining a sufficient number of cells for the procedure, problems with the culture and storage of the cells, and poor overall engraftment of the cells. It is clear that alternative therapies are needed.

A.2. Embryonic stem cells as alternative therapy

Stem cells have long been of interest in the field of cell therapy and regenerative medicine. There are two major categories: embryonic and adult stem cells. Embryonic stem cells are derived from totipotent cells of the inner cell mass of the human blastocyst, and are capable of unlimited in vitro proliferation as undifferentiated cells. In addition, these cells can eventually differentiate down multiple tissue lineages (2, 3). Better understanding of the totipotent capabilities of embryonic stem cells and the elements driving their differentiation

to various tissue lineages could lead to dramatic advances in the treatment of various diseases. Because of their early human origins, though, research and use of embryonic stem cells has been hotly debated in politics as well as the public. It is a topic with many legal, moral and ethical issues. In addition, administration of human embryonic stem cells may cause tumor formation (4); thus any potential therapies would need to assure that division of transplanted cells could be controlled.

A.3. Adult stem cells as alternative therapy

The use of adult stem cells for research and treatment is less politically and emotionally charged since it does not require the destruction of embryos. Adult stem cells are found in many adult tissues including blood, intestine, neurons, skeletal muscle, and skin, and support cellular renewal of the tissue to which they belong (5-9). Traditionally, it was thought that only embryonic stem cells could differentiate into adult cells of more than one lineage. Observations calling for adjustments of this view include studies showing that bone marrow-derived progenitors participate in regeneration of damaged skeletal muscle (10), liver cells (11, 12) , ischemic myocardium (13-15), as well as participate in neurogenesis (16, 17). Neural stem cells were shown to participate in hematopoiesis (18), and furthermore, to contribute to cells types of all three germ layers (19). These findings suggest that adult stem cells possess the ability to “transdifferentiate”, or differentiate into cell types outside of their specific tissue lineage. This remarkable concept, also termed cellular “plasticity”, calls into question not only our traditional views on the role of the adult stem cell, but also the entire tri-laminar view of embryonic development.

Because of their newly discovered pluripotent abilities, including the ability to become hepatic cell types, and their relative lack of ethical and legal issues, these cells may

be optimal candidates for use in treatment of various liver diseases. However, the concept of plasticity is not free of controversy. Of note, all of the data has not been universally confirmed, with some research groups unable to reproduce such findings and other groups suggesting mechanisms other than transdifferentiation, such as cell-cell fusion, as the responsible phenomena (20-22). The arguments against plasticity and the concept of fusion will be discussed in more detail in section A.11. and A.12, respectively.

A.4. The bone-marrow and its stem cells

Bone marrow has two components. The first component is a highly organized stromal component that serves as a backbone on which the second component, the cells of the hematopoietic system, grow and differentiate. There are at least two stem cell types in the bone marrow: mesenchymal stem cells, which give rise to osteoblasts, chondrocytes and other cell types, and hematopoietic stem cells (HSC) which give rise to cells of the hematopoietic system (23).

Functional definitions make it difficult to identify stem cells for research

Plasticity research involving bone-marrow stem cells include experiments using both whole bone marrow, as well as specific bone marrow stem cell populations. Finding a reliable way for researchers to identify adult stem cells has been difficult, as these cells are traditionally defined by their functional attributes and not by directly observable markers. Adult stem cells are defined as a potentially heterogeneous population of undifferentiated cells (relative to a functional tissue), capable of proliferation, production of a large number of differentiated functional progeny, self-maintenance of their population, regeneration of the tissue after injury, and flexibility in use of these characteristics (24, 25). Functional definitions, while the gold standard for reflecting biological roles, create a conundrum in the

identification of stem cells. Identification would require functional testing, including differentiation down various lineages. One would potentially alter the cells original properties and render them no longer useful in experimentation (25). Our current definitions based on phenotypic markers, gene expression profiles, and other molecular definitions miss essential functional aspects of these cells, but allow researchers to sort and isolate cells.

Markers for identification of hematopoietic stem cells and their subpopulations

At present, cell-surface markers are used to identify stem cells. These markers sometimes differ in rodents and humans. In mice, HSC have been defined by the absence of markers of hematopoietic differentiation. This profile has been termed “lineage negative” (Lin-). In addition, the cell should include low expression of thy-1 (thy-1^{lo}), and high expression of c-kit and sca-1 (26). HSC can be obtained for use either through harvesting of bone marrow or by stimulating their mobilization into peripheral blood via granulocyte-colony stimulating factor (G-CSF) administration and subsequently collecting the blood via phlebotomy. In humans, the major marker for HSC is CD34 antigen (27). CD34 can not be used as a reliable marker in murine studies, however, as the expression of CD34 is rather dynamic in the mouse and presence or absence of the marker may not reflect whether the cell is a stem cell (28). For humans, though, it is a very reliable marker, and as a result, the presence of CD34 is used to identify the population of cells used in blood stem cell transplants in human patients. Antibodies used to identify $CD34^{+}$ human cells also reveal subpopulations. AC133 is a monoclonal antibody that identifies the CD133 antigen on some cells in what is termed a $CD34^{bright}$ subpopulation. This subpopulation has been shown to have greater hematopoietic reconstituting properties than the $CD34^{dim}$ population in xenotransplantation models (29). In addition to $CD34^{+}$ populations, $CD34^{-}$ populations have

also been found to have stem cell properties. In mice, staining HSC with the fluorescent, DNA-binding dye Hoechst 33342, identifies a side population (SP) of $CD34^-$ cells recognized to be a primitive group of stem cells, and is described as $CD34^-$/low, c-Kit^+, Sca-1^+ (30). It was furthermore shown that this SP phenotype was characterized by the expression of the ATP-binding cassette (ABC) transporter subtype: ABCG2 (31, 32). ABC transporters have been shown to be upregulated not only in HSCs, but also in oval cells (intrahepatic progenitors capable of producing hepatocytes and cholangiocytes) of both rodents (33), and humans (34). This would indicate an overlap of surface markers in different tissues, showing that various adult tissue stem cells may be more similar than originally thought, and making it more difficult to distinguish between stem cell groups in areas, such as the liver, where stem cells from different tissues can mix.

Hematopoietic stem cells and liver progenitor cells share molecular markers

Interestingly, oval cells, express many factors in common with hematopoietic progenitor cells such as: c-kit^+, $CD34^+$, Thy-1^{lo}, and Sca-l^+ (35, 36). Moreover, the location that some investigators have found oval cells, the periductular/intraportal zone in the liver, also suggests that they may originate from an extrahepatic source, possibly bone marrow. These similarities give more credence to the idea that adult tissue stem cells are not lineage-restricted, and specifically that HSC could become oval cells in the liver. It has been demonstrated that oval cells can arise from bone marrow derived cells (11), discussed further in A.6., though other investigators have not been able to reproduce these results (37, 38).

Markers for mesenchymal stem cells and their subpopulations

Mesenchymal stem cells are known to contain a subpopulation of stem cells known as multipotent adult progenitor cells (MAPC). MAPC have demonstrated a surprising degree of

plasticity (39), as have HSC (40) (see section A.6.). In-vitro, under specific culture conditions, MAPC have been shown to differentiate into multiple epithelial cell types including hepatocytes (39, 41). They can be cultured from human, mouse and rat bone marrow, and do not express, or express only low levels of, the CD44 antigen (a marker for mesenchymal stem cells) (39, 42). MAPC are CD105 (also called endoglin, or SH2) negative (43) and are unlike HSCs in that they do not express CD34 or cKit, but are like HSCs in that they express Thy1, AC133 (human MAPC) and Sca1 (mouse), albeit at low levels (42, 44). In the mouse, MAPC express low levels of stage specific embryonic antigen (SSEA)-1, and low levels of the transcription factors Oct4 and Rex1. These are known to be important for maintaining embryonic stem cells as undifferentiated and are down-regulated when embryonic stem cells undergo somatic cell commitment and differentiation (45).

Bone marrow may contain stem cells from other tissues

While much work has been done in identifying the various populations of bone marrow stem cells, many experiments have been performed with unfractionated bone marrow. Recent studies suggest that the bone marrow compartment harbors adult stem cells from other tissues of the body (46), such as liver and muscle. The stromal-derived factor-1 (SDF-1)-chemokine receptor 4 (CXCR4) axis, which acts as a powerful chemoattractant for HSC, could also have the same chemoattractive effects on early tissue committed stem cells (TCSC) not of bone marrow origin. The authors found a highly mobile population of CXCR4+ cells expressing mRNA/proteins for various markers of early TCSC. They postulate that the bone marrow is not only a home for HSC, but also a 'hideout' for non-hematopoietic CXCR4+ TCSC (46). These data underline the need for unambiguous markers of various tissue stem cells in plasticity research.

A.5. The liver and its regenerative abilities

The adult liver is remarkable in its ability to regenerate after injury. Mild to moderate liver injuries are repaired via rapid proliferation of existing mature hepatocytes. Experiments involving partial hepatectomy (47) or carbon tetrachloride (CCl_4) toxicity (48) show that liver regeneration can occur by mature hepatocyte proliferation alone. When liver injury either surpasses the regenerative capacity of the hepatocytes or causes hepatocyte regenerative capacity to be impaired, liver progenitor cells (oval cells) differentiate into hepatocytes and biliary epithelial cells (cholangiocytes) (49-53). Models of severe injury such as CCl_4 combined with 2- acetylaminofluorene (2-AAF), a hepatocarcinogen that inhibits mature hepatocyte proliferation, show that large numbers of small oval cells appear (54). Subsequent studies showed that these small oval cells were able to differentiate into hepatocytes and ductular cells (55), suggesting that these cells indeed are an intrahepatic stem cell, and that these cells divide in response to injury overwhelming to mature hepatocytes. Another model of severe injury involves administration of retrorsine, a DNA alkylating agent that is selectively taken up and activated by hepatocytes to inhibit their proliferation, in addition to CCl_4. The cells responsible for regeneration in this model were progenitors expressing phenotypic characteristics of oval cells, but the cells were found to be morphologically unique from oval cells (56). One model for liver regeneration suggests that liver regeneration happens on three levels: by way of hepatocytes, intrahepatic stem cells, and extrahepatic stem cells (57). Despite these extraordinary abilities, however, this remarkable regenerative capacity can be superseded, as evidenced by various diseases that destroy the liver.

A.6. Bone marrow stem cells become liver cells

In light of the new found plasticity of adult stem cells, a number of investigators have begun to examine how bone marrow stem cells might be used to rebuild damaged liver. Trying to understand the processes controlling the ability of these cells to enter other tissues and contribute to various cell types has proved difficult and complex, and is not well understood.

Early studies show bone marrow plasticity in damage models

The first demonstrations of bone marrow-to-liver plasticity employed either sex-mismatched or strain-mismatched donor and recipient rats. Male bone marrow was used to rescue lethally irradiated female rats, establishing bone marrow chimeras in which marrow-derived cells could be identified by the presence of the Y chromosome. Similarly, bone marrow from wild-type donors was transplanted into dipeptidyl peptidase (DPPIV)-deficient rats, allowing DPPIV to be used as a marker of bone marrow origin. In each case, liver injury was induced after stable engraftment of donor bone marrow cells. Thus, the bone marrow transplant was performed to mark the bone marrow, and was not intended to have any therapeutic impact on the liver. 2-AAF was administered to intentionally suppress regeneration from mature, native hepatocytes. In addition, CCl_4 was given to produce acute hepatic injury and regeneration including oval cell upregulation. After liver injury, Y chromosome positive (male) hepatocytes were found in female animals, and in DPPIV deficient rats, DPPIV positive hepatocytes and oval cells were found (11). This showed that marrow-derived stem cells could act as hepatic cell progenitors in a model of impairment of the hosts own regenerative capabilities.

Bone marrow plasticity in models lacking injury

The potential plasticity of HSC, rather than whole marrow, was evaluated by transplanting single, male, lineage-, HSC into irradiated female recipients (along with supporting female donor cells). In addition to complete bone-marrow reconstitution, male hepatocytes and cholangiocytes, and male epithelial cells throughout the gastrointestinal tract, bronchus, and skin were found by colocalization of fluorescence in situ hybridization (FISH) for Y chromosome and immunohistochemistry (IHC) for various epithelial markers (40).

Additional studies demonstrated marrow-derived hepatocytes in the absence of liver damage. Male bone marrow was infused into lethally irradiated female mice and allowed to stably engraft for 6 weeks. Detection of donor and host-specific phenotypes was performed by FISH for Y chromosome, and albumin mRNA detection for hepatocytes. A significant number of donor-derived (Y chromosome positive) hepatocytes were found by co-localization of the two labeling techniques (12).

A.7. Injury vs. non-injury models of plasticity

In multi-organ engraftment studies, the highest levels of donor-derived cells, as well as the most diffuse clustering of donor-derived cells, were seen in alveolar epithelium (39). It had been postulated that the higher level of engraftment in this tissue is likely related to the fact that lung tissue is known to be more radiosensitive than other tissue (58). Therefore the increased injury induced by irradiation may have been responsible for the increased levels of engraftment. The relevance of liver irradiation to transdifferentiation in hepatic models is less clear because while liver damage via irradiation is known to occur (59), it tends to be seen at larger doses than that delivered during bone-marrow ablation (1000-1200 rads).

Neither of the two studies showed evidence of tissue damage, histologically, leading researchers to consider the possibility that marrow-derived hepatocytes may occur in minimal liver injury, or even in physiological maintenance. At appropriate doses, liver irradiation can decrease the hepatocyte's own ability for regeneration (60), and therefore may be a useful model for studying injury and its effects on marrow-derived engraftment in the liver.

Injury increases engraftment of hepatocytes by bone marrow

Regardless of injury method, studies with moderate to severe liver damage tend to show increased hepatic engraftment of marrow cells. One study employed an in vivo selection strategy, based on the protective effect of Bcl-2 against Fas-mediated cell death, to confer a survival advantage to the progeny of transplanted bone marrow cells. Donor marrow was harvested from mice over-expressing the Bcl-2 gene under the control of a liver-specific promoter and transplanted into normal mice. The mice were then injected repeatedly with Fas-agonist antibody to induce severe apoptotic liver injury, while the control group did not receive Fas-agonist antibody. After multiple cycles of apoptotic injury and recovery, mice that received the Fas-agonist antibody developed clusters of mature marrow-derived hepatocytes expressing Bcl-2 (61). This study helped to show that while transdifferentiation under physiological conditions may be an insignificant event, liver injury and in vivo selection may upregulate this process.

In a model of CCl_4-induced liver fibrosis, GFP^+ bone marrow cells from donor mice contributed to approximately 26% of the recipient liver 4 weeks post transplant (62). However, the extraordinarily high percentage of engrafting cells has been criticized for ambiguous identification methods and unusual architecture of the GFP-expressing cells (63).

Damage seems to increase engraftment, but not in predictable ways

Presence and severity of liver injury are likely important in stem cell plasticity and engraftment, yet there is large variation in the reported extent of plasticity via severe liver damage models. Estimates of the percentage of marrow-derived cells range from zero percent (64), to nearly 30% hepatic repopulation at 4 weeks post transplant (62). One possibility for these observed differences may lay in the ability of different subpopulations of adult stem cells to differentiate into functional tissues. This cause of divergence likely plays a role in not only damage studies, but non-damage studies as well, and suggests a need for researchers to define groups of marrow stem cells before their experimental use. This and other possible reasons for variations in different results are discussed in section A.11.

A.8. Do Adult Tissue Stem Cell-Derived Hepatocytes Have Functional Significance?

The studies above suggest that marrow-derived stem cells can contribute to hepatic regeneration, yet the ability to apply this phenomenon in medicine would depend on whether these engrafted cells have sufficient function. Testing for function in marrow-derived hepatic cell types has proven difficult. Studies with the most success use models where recipient animals are mutant, lacking normal cell function in the tissue being studied, and donor animals are wild type for this characteristic. In this way, donor cells restore function to recipients. Functional analysis was carried out in experiments with fumarylacetoacetate hydrolase (FAH) deficient mice. This is a model of fatal hereditary tyrosinemia liver disease. Wild-type bone-marrow was transplanted into lethally irradiated FAH^- mice. FAH^- mice will develop progressive liver failure unless treated with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) to prevent accumulation of toxic catabolites of tyrosine. Post bone marrow transplantation, NTBC feedings were cyclically halted,

creating liver damage and therefore selecting for liver-repopulating conditions. After euthanasia, up to 30-50% of liver mass was bone-marrow derived. In addition, large donor-derived nodules consisting of morphologically normal, FAH^+ hepatocytes were visualized. Biochemical functional assessment of the marrow-derived cells revealed expression of liver hydrolase (previously missing in the FAH^- recipients) to nearly normal levels. This led to long-term survival of the animals without a need for NTBC feedings (65). Therefore in rodents, it appears that HSC have the ability to contribute to functional liver repair in a model of liver injury where there is a survival advantage of the infused stem cells. Subsequent analysis of marrow-derived hepatocytes in this model suggested that some, if not all, of the resulting functional hepatocytes may be a result of fusion between diseased native hepatocytes and donor-derived cells of the monocyte-macrophage lineage. The role of fusion will be discussed in detail in part A.12.

A.9. Plasticity of bone marrow cells has also been found in humans

The establishment of a role for bone marrow stem cells in engraftment and repair in various tissues other than bone marrow in mouse studies has been an important first step in understanding how to better use the capabilities of these cells in human disease. Therapeutic clinical use of such technology, however, would only be possible if there is evidence that human adult stem cells also have the plastic capabilities seen in adult rodent stem cells. The first reports that hinted at transdifferentiation in human cells occurred with archival biopsies. Liver specimens from recipients of sex-mismatched bone marrow or liver transplants were analyzed to look for marrow-derived hepatic cells. FISH for the Y chromosome as well as IHC for markers in liver cells was employed to analyze liver and bone marrow samples. Y-positive hepatocytes were found in female patients who had received male bone marrow

transplants and y-positive hepatocytes were also found in female livers that had been transplanted into male recipients (66), suggesting the engraftment of a circulating progenitor in the liver of the recipient. Marrow-derived hepatocytes and epithelial cells of the skin and gastrointestinal tract were seen in patients who had first received myeloablative therapy, and then received an infusion of peripheral-blood stem cells for the treatment of hematological malignancy (67). This showed that marrow-derived progenitors circulate in the blood and contribute to non-hematopoietic tissues in humans.

One concern that arose from these studies was that perhaps the Y chromosome positive hepatocytes were not the result of transdifferentiation, but instead a result of transplacental passage of male fetal progenitor cells during pregnancy (68). Fetal-maternal microchimerism has been documented (69, 70), but can not be completely responsible for y-positive hepatocytes in the previous studies. Marrow-derived cells have been seen after bone marrow transplantation in human liver from nulliparous females (66, 68) and in a female with no history of male child-bearing (71). In murine models, female recipients were nulliparous (12).

Increasing injury correlates with increasing engraftment in archival biopsy studies

Further analysis of human archival biopsy tissue showed that increasing severity of liver injury correlates directly with hepatic engraftment frequency. Human liver allograft biopsies revealed varying degrees of liver injury ranging from mild biliary obstruction in most to fibrosing, cholestatic hepatitis in one. Hepatic engraftment frequency was seen to increase with increasing liver injury (66). Contrary to this, recipients of bone marrow transplantation who did not have hepatic injury demonstrated hepatic engraftment despite the lack of liver injury (66), in keeping with the results found also in murine studies (12). This

suggests, as in the animal data, that there is a role for adult tissue stem cell engraftment both in physiological maintenance as well as in acute injury.

In-vivo evidence of plasticity and functionality of derived cells with human adult stem cells

While archival biopsies have revealed much information, it is clear that better models for studying human cells in-vivo are needed. One report studied in-vivo physiological maintenance roles of tissue stem cells. Human cord blood cells were injected into sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, and human-derived hepatocytes were found in murine liver (72). In a similar study, these human-derived hepatocytes were found to produce human albumin mRNA, detected by reverse transcription-polymerase chain reaction (73).

To study in-vivo engraftment of human cord blood cells in a model of hepatic injury, hepatic injury was induced via one-third partial hepatectomy and 2-AAF in mice. Functional human-derived hepatocytes were found (74). Comparison between a group of mice with CCl_4 induced liver injury and a non-injured groups of mice showed that only mice who had been administered CCl_4 in addition to receiving a $CD34^+$ fraction of blood were found to express human albumin (75).

A.10. Homing mechanisms of bone marrow stem cells to liver: SDF-1/CXCR4 axis and hepatocyte growth factor help bone marrow migrate to liver and become hepatocytes

Study of the stromal-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) axis has given insight on how HSC migrate to damaged liver. The SDF-1/CXCR4 axis was shown to regulate movement of HSC toward damaged liver in a NOD/SCID model (76). In addition, hepatocyte growth factor, a substance that is upregulated in liver injury (77) and has been shown to help HSCs differentiate into hepatic cells (75), was shown to be a key player

in the recruitment of stem cells. This was found to occur through hepatocyte growth factor's interactions with SDF-1 (76).

A.11. Plasticity is not supported universally in the literature

While the concept of plasticity is one that conjures excitement and hope for the field of regenerative medicine, it has been questioned by some groups who have been unable to reproduce similar findings. No evidence of epithelial engraftment at physiological conditions was seen in parabiotic mice, in which two mice, one GFP^+ and the other GFP^-, shared a common circulation. Under these conditions, with no specific injury, cross-engraftment of hematopoietic tissue was found, but no engraftment of non-hematopoietic tissues was discovered (20). In another model, no significant hepatic engraftment was found after sex-mismatched bone marrow transplant (78).

Reasons for variation in findings among studies

The negative results of these experiments may be attributed, at least in part, to model systems chosen. For example, GFP may exhibit weak and variable expression in epithelial cells of the donor animal. Other reasons for the variations in results may be age of donor mice, length of time after transplantation that tissues are examined, purification techniques during isolation of stem cells, and methods used for detection of donor-derived cells. These reasons are explored in detail in a recent commentary (79). The population or subpopulation of stem cells used in experiments may also play a role. While initial studies used unfractionated bone marrow, newer work showed the capacity of $CD34^+$ cells, specifically, for hepatic engraftment (12). The SP fraction of bone-marrow cells, discussed earlier as a

CD34$^-$ primitive population (28, 30), was also demonstrated to contain cells with ability for hepatic engraftment (80).

A.12. Fusion could to play a role in observed plasticity

Other work has challenged the concept of plasticity by suggesting that some or all putative transdifferentiation events are instead the result of cell-cell fusion (between donor-derived blood cells and existing differentiated cells). An in vivo study infused wild-type male marrow cells into female FAH$^-$ mice. FAH expressing liver nodules were found, and genomic analysis of the DNA dissected from the liver nodules was performed. The DNA was probed for FAH sequences and Y chromosome sequences to measure the proportion of donor alleles. Hepatic nodules contained more mutant than wild-type FAH alleles, and nodal hepatocytes expressed both donor and host genes. The authors found this to be most consistent with polyploid genome formation by fusion of host and donor cells (81). Another study, also using wild type male donor marrow into FAH$^-$ female mice, performed serial transplantation of bone marrow. Genomic DNA from bone marrow derived hepatocytes from the tertiary recipients was analyzed. While massive liver repopulation had occurred, only a very small amount of the original donor genotype was preserved in the cells, far less than expected if the cells had come solely from transdifferentiation of HSCs (65). Another group employed karyotype analysis to address the fusion issue. Wild type bone marrow from female mice was injected into male FAH$^-$ mutants, and chromosomes from the metaphase part of the cell cycle were analyzed. Control nuclei and up to half of the derived hepatocytes had the expected normal male diploid (56, XY) or tetraploid (81,XXYY) karyotype. Most of the rest of the cells had karyotypes consistent with donor-recipient fusion. In addition, only a small amount of cells contained only X chromosomes, revealing that the original donor

female karyotype had been lost, also consistent with fusion (80). Other investigators created models based on the normal ploidy values for murine hepatocytes that predicted the percentage of donor DNA that should be present in regenerated nodules if fusion had occurred and if it did not occur. Each of the nodules analyzed yielded values outside the predicted range for transdifferentiation alone (81).

It is known, however, that conditions of liver injury and impairment of DNA repair mechanisms (such as via ionizing radiation) can cause advanced hepatic polyploidy as well as terminal differentiation and cell senescence (82, 83). Some suggest that hepatic polyploidy may come from fusion of multinuclear cells and may account for some of these results. Besides liver, there have been reports of fusion of bone marrow cells with cardiac myocytes and purkinje neurons in vivo (22).

If fusion accounts for the observed events, it is necessary to determine which cells are fusing with host liver cells. It could be the donor HSC, but it has also been suggested to be hematopoietic progeny from the HSC, including macrophages, also known as Kupffer cells in the liver (81). Macrophages are likely candidates due to their large number, location, and because they tend to fuse with cells under other conditions (84). In fact, it has been suggested that cell plasticity can be explained by donor HSC homing to the bone marrow and creating myeloid cells that then can fuse with recipient tissue cells (85). Liver injury could call upon the circulating myeloid cells, descended from donor HSC, as part of an inflammatory response to injury. Inflammation may create a fusogenic environment by facilitating myeloid cell recruitment to liver (or other tissue) where subsequent fusion with the parenchyma can occur. Experimentally this scenario has been demonstrated by transfusing lymphocyte-deficient cells, then mapping the fate of the myeloid lineage. HSC-

derived hepatocytes were found to derive primarily from myeloid cells, indicating that they are the cells responsible for fusion (86).

Some models of plasticity are not consistent with fusion

Other studies show that fusion does not account for bone marrow plasticity. In xenogeneic studies where human cells are transfused into rodent recipients, FISH analysis of human and murine DNA as well as nuclear staining patterns showed no evidence of fusion between cells (72, 73). Researchers transplanted lethally irradiated female mice that ubiquitously expressed Cre recombinase with bone marrow from male Z/EG Cre-reporter donor mice. In this model, cells resulting from fusion of a bone marrow derived cell with a host cell should express enhanced green fluorescent protein (EGFP), and would therefore be distinguishable. When analysis of tissues was performed, Y chromosome positive, recipient epithelial cells were noted without EGFP expression. Therefore no fusion was observed in bone-marrow derived epithelial cells(87).

A novel in-vitro model system aimed at discriminating between fusion and transdifferentiation involved the co-culturing of murine HSC with either normal or damaged liver tissue. The HSC and liver cells were separated by a trans-well membrane. In this system, the cells would not be allowed to fuse since there was no contact between the HSC and liver cells. Immunofluorescence showed that the HSC co-cultured with damaged liver lost their hematopoietic phenotypes as they began to express albumin. Tissue-specific markers normally expressed during liver differentiation were also detected providing further evidence of the HSC to hepatocyte conversion. Cytogenetic analysis of the cells gave further assurance. The model system was such that the HSC were male cells and the liver tissue was from female donors. Analysis revealed tetraploid cells amongst the HSC, though the

karyotype was XYXY and therefore only derived from male cells, HSCs with other HSC, and not from HSC fusion with female liver cell (XXXY). The investigators concluded that there is genuine plasticity of HSC cells and that HSC transdifferentiation is an early event (in contrast to the studies above) with microenvironmental cues responsible for the germ-layer switch (88).

FAH model systems, fusogenic environments and normal hepatocyte polyploidy could account for both real and apparent fusion outcomes

In addition to the above evidence against fusion as the sole mechanism for apparent plasticity, there are issues that have been found with the FAH-null model (a key model responsible for suggesting fusion as a cause for donor derived hepatocytes). It has been suggested that since in the FAH-null model there is extreme architectural disruption and membrane instability of hepatocytes, this environment may lend itself more to fusion (89). In addition, hepatocytes are known to fuse in pathological conditions.

Important to note is that hepatocytes can also be multinucleate and polyploid under normal conditions. Hepatocytes are tolerant of high ploidy without any evidence of malignant transformation. This should be considered when DNA and karyotype analysis is employed to determine if fusion has taken place.

Fusion and plasticity may occur together

Reports supporting stem cell plasticity (87, 90, 91), as well as convincing in-vitro HSC plasticity accounts (86) make it difficult to attribute plasticity to fusion alone. While fusion may well be occurring, it seems unlikely that it is the sole mechanism for engraftment. Investigators reporting multipotent adult cells derived from adult human bone marrow (39) that were able to differentiate in vitro into a number of mature cell types including functional

hepatocyte-like cells (41), suggest true adult cell plasticity, at least in vitro. Cell fusion may occur in some systems, while transdifferentiation with or without fusion may occur in others. At present, the factors regulating this are unclear and need to be better understood before these phenomena, both plasticity and fusion, can be of clinical use.

B. Hypotheses and Study Aims:

The liver is extraordinary in its ability for intrahepatic cells to proliferate and differentiate in response to liver damage. Hepatocytes and liver progenitor cells, oval cells, can both contribute to regeneration depending on types and severity of injury. It has recently been found, however, that human as well as mouse blood and bone marrow can also give rise to hepatic cell types (40, 66, 67, 75). The mechanisms by which this seemingly plastic event takes place are still to be determined. Learning more about the process by which these blood and marrow cells are able to contribute to hepatic cell types, and facilitating the transformation to a high enough level could enable us to better utilize this phenomena in the cellular therapy of various liver diseases. Reports on the plasticity of blood and bone marrow cells indicate that the process may be one occurring physiologically or may be upregulated by liver damage and can occur without fusion (73, 87, 88). These reports suggest a degree of phenotypic plasticity of adult cells that has largely been unrecognized.

B.1. Hypothesis: Oval cells can arise from bone marrow and can engraft the liver as epithelial cells. Furthermore, since oval cells are presumed to be the stem cells for the liver, engraftment of marrow derived cells into oval cells will ultimately lead to marrow-derived hepatocyte and cholangiocyte progeny of the engrafted oval cell.

B.2. Aims of the Study:

Aim 1: Induce oval cell proliferation in male/female bone marrow chimeras using DDC.

By feeding 3,5 diethoxycarbonyl-1,4 dihydrocollidine (DDC), a toxin known to cause cholestatic liver injury and to increase oval cell proliferation, to male into female bone marrow chimeric mice we hope to induce severe, but reversible, injury to the liver with upregulation of oval cell proliferation. We hope to determine if the DDC-damage induced hepatocytes and cholangiocytes are, at least in part, marrow-derived. In addition, by using the DDC damage model we hope to be able to learn more about the temporal relationship of the appearance of marrow-derived hepatocytes to liver damage and recovery

Aim 2: Inhibit hepatocyte proliferation by focal irradiation of the liver.

By treating mice with liver-specific irradiation, we hope to further suppress the regenerative capabilities of the cells within the liver so that we may enhance the homing and engrafting of marrow-derived cell types into hepatic cell types. We will compare this to a group of mice not treated with liver-specific irradiation to learn more about types of damage and their effects on marrow-derived cell engraftment in the liver.

C. Materials and Methods:

C.1. Stem cell isolation and transplantation

To isolate bone marrow cells, 6 week old, male, C57Bl6 mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with isoflurane (Abbott Labs, Chicago IL), sacrificed by cervical dislocation and hind limbs were removed. Bone marrow was flushed with a 25 gauge needle in sterile fashion from the medullary cavity of the tibias and femurs with medium containing IMDM + 5% FCS. This technique has an approximate yield of 10×10^6 cells per mouse. 1.0×10^6 male mononuclear bone marrow cells were injected into the tail veins of 20 female C57Bl6 mice previously treated with a lethal dose (1100rads) of whole body irradiation in a cesium irradiator. Transplanted marrow was allowed to engraft for 6 weeks.

C.2. Liver Irradiation and DDC exposure

12 of the female bone marrow transplanted mice received focal irradiation (Siemens stabilipan x-ray source) of 1000 rads, limited by lead, to the upper abdomen to inhibit hepatocyte regeneration from existing hepatocytes. All mice (those treated with focal irradiation as well as those who were not) were then fed for 10 days with a 0.1% DDC (Research Diets Inc) diet to stimulate oval cell proliferation. Mice were sacrificed by isoflurane at 0, 7, 14, 21, 42, 63, and 84 days following the last dose of DDC.

C.3. Tissue Processing

Portions of liver and spleen were collected, fixed in phosphate buffered formalin at room temperature for 4 hours, and then embedded in paraffin. Three micron sections were cut for further analysis.

C.5. Immunohistochemistry and FISH Analysis

Slides were deparaffinized via heating and Citrisolv treatment, and rehydrated through graded alcohols to phosphate buffered saline (PBS). Antigen retrieval (a method of recovering the antigenicity of tissue sections that has been masked by formalin fixation) was carried out with Retrieval-All-2-pH10 (Signet Labs, Dedham, MA) for 30 min in a steamer. Slides were then cooled, washed in PBS, then water, and air-dried. A digoxigenin-labeled mouse Y chromosome probe (Prepared as in Krause et al., Cell Vol. 105, 369-377, 2001) was applied. Coverslips were sealed with rubber cement and DNA was denatured at 60^0C for 6 minutes. Following an overnight hybridization at 37^0C, slides were washed in 0.2X SSC for 3 minutes at 42^0C. Non-specific protein binding was blocked with 2XSSC, 3%BSA, 0.1%NP40 for 30 minutes. Detection of the Y chromosome was performed by adding anti-digoxigenin rhodamine (Roche)(1:10), in 4XSSC, 1%BSA, and 0.1% T20 for 1 hour at 37^0C. Slides were washed in PBS, and a 1:25 dilution in PBS-1% bovine serum albumin (BSA) of rabbit anti-human polyclonal antibody to hepatocyte nuclear factor (HNF-1) (Santa Cruz Biotechnology) was applied overnight at 4^0C. Following PBS washes, the secondary antibody, goat anti-rabbit FITC (Invitrogen)(1:50 in PBS/1%BSA), was incubated on the slides for 1 hour at 37^0C. Slides were washed in PBS, air-dried, and mounted in vectashield fluorescence mounting media with DAPI (Vector Labs, Burlingame, CA).

C.6. Tissue analysis and Cell Counts

Counting of y-positive, HNF-1-positive hepatocytes was accomplished by systematically examining the treated slides, field by field, under 40x magnification, using an Olympus BX-51 microscope. Y-FISH and HNF-1 double positive cells are identified using a dual-wavelength channel filter set designed for simultaneous visualization of FITC and Rhodamine (Chroma Technology). Photomicrographs were taken using a digital camera and IPLab imaging software (BD Biosciences, Rockville, MD). Separate images were obtained using filters for DAPI (all nuclei), rhodamine (Y chromosome), and FITC (HNF-1), then merged. Cell counts were derived from these images. Hepatocytes were identified by HNF-1 nuclear staining, and also histologically as large polygonal cells, arranged in plates, with large, round nuclei. Select hepatocytes, with both HNF-1 and Y chromosome labeling, were further analyzed by confocal microscopy (Zeiss LSM 510 microscope). Hematoxylin and eosin stained sections of each sample were also prepared, and light microscopic photographs were taken on the Olympus BX-51 microscope in order to examine the histology of the liver samples and various liver damage.

C.7. Statement of animal care

This research proposes the use of liver injury and bone marrow transplant models to determine the mechanism of liver engraftment by bone marrow stem cells. The liver injury models have been approved by the Yale Institutional Animal Care and Use Committee (IACUC) (2004-10416, 3/3/2004). Bone marrow transplantation is also approved under this protocol. Animals were cared for in a humane manner in accordance with IACUC standards.

C.8. Statement on persons completing various portions of labor

Bone marrow isolation and transplantation, liver irradiation and DDC exposure, animal sacrifice, tissue processing, immunofluorescence and FISH analysis, were all performed by this author as well as Scott Swenson. Tissue analysis and cell counts were in the vast majority performed by Scott Swenson, with minor analysis contributed by this author. Confocal microscopy was done by Scott Swenson. Labeling of paraffin tissue sections with hematoxylin and eosin was done by the Yale Research Histology Lab. Images from hematoxylin and eosin stained sections were gathered by this author. The thesis was written by this author.

D. Results:

D.1. Experimental design

To determine whether bone marrow cells differentiate into liver progenitor/oval cells, lethally irradiated female mice were reconstituted with bone marrow from male donors. This transplantation served to mark the cells of marrow origin. The donor marrow was allowed to stably engraft for six weeks. Reconstitution using this protocol typically replaces >80% of the recipient bone marrow with donor marrow. Subsequently, the mice were treated with DDC diet for 10 days in order to cause acute liver injury and stimulate the activation and proliferation of oval cells. A subset of the bone marrow chimeric mice were also subjected to liver-specific irradiation on day 0 of the DDC diet for the purpose of suppressing hepatocyte proliferation after injury and favoring mobilization of potential progenitor cells from outside the liver. The experiment design is summarized in Figure 1.a. The apparatus for liver irradiation is shown in Figure 1.b. Control mice received no post-transplant injury. Mice were sacrificed at 0, 1, 2, 3, 6, 9, and 12 weeks after completing a 10-day course of DDC diet, with or without liver irradiation. These points were selected in order to monitor the appearance of marrow-derived hepatic cells over time.

D.2. Histological changes

Paraffin sections of control and experimental animals were stained with hematoxylin and eosin in order to observe histological changes induced by DDC alone, or DDC plus liver specific irradiation, as well as to observe histological recovery from these injuries over a period 0-12 weeks post completion of the 10 day DDC diet (Figure 2). Normal murine liver is shown in Fig. 2a. Fig. 2b shows liver from a control (bone marrow transplant without liver injury) mouse. Both the normal and control liver sections show no apparent abnormalities.

In both groups, histological changes were seen at 0, 1, 2, 3, 6, 9, and 12 weeks (Figure 2c-e) after completion of the DDC diet. Ductal proliferation, inflammation involving both oval cells and inflammatory cells, and cholestasis can be seen in both experimental groups at all time points of recovery. Histologic recovery from DDC injury was delayed by liver irradiation.

D.3. Biochemical injury

Blood samples were taken from experimental mice at 0,1,2,3,9 weeks post DDC/DDC and liver specific irradiation injury, to analyze for biochemical injury and recovery, and to better understand which parts of the liver have been affected by the inflicted damage. Elevation of alanine aminotransferase (ALT) above normal levels (50 - 160 units/L) is a measure of hepatic cell inflammation, while elevation of alkaline phosphatase above normal levels (1 - 21 units/L) indicates inflammation of the biliary tract cells, cholangiocytes. The DDC-induced biochemical injury, shown in Figure 3, was reversible in both the irradiated and non-irradiated group, and did not appear to be worsened by hepatic irradiation with 1000 rads. Both ALT and AP show large elevations from the normal range, peaking at the end of the DDC treatment period (0 weeks recovery) and subsequently declining toward normal.

D.4. Y chromosome positive cells in the liver of recipient animals

The identification of marrow-derived hepatic cells requires the simultaneous detection of markers for both donor and recipient cell phenotypes. The Y chromosome is a useful marker of donor origin because it is unique to our donor animals and is readily detected in the nucleus by FISH. Hepatocyte Nuclear Factor-1 (HNF-1), a transcription

factor highly expressed in the nucleus of hepatocytes, cholangiocytes and oval cells, is a useful marker of recipient origin because it is not expressed in any cells of blood lineage, and can be easily detected by immunofluorescence. In addition, HNF-1 is also expressed in epithelial cells of the intestine, pancreas and kidney. Within the liver, HNF-1 is not expressed in endothelium, stellate cells, or fibroblasts.

By employing a double label FISH/immunofluorescence protocol, we were able to detect both Y chromosome and HNF-1 in formalin-fixed, paraffin embedded tissues of the liver. To be considered a marrow-derived hepatocyte or cholangiocyte, a given cell was required to have both Y chromosome and HNF-1 unambiguously in its nucleus. Having both markers in the nucleus of the target cell ensures less chance of mistaking a marrow derived hepatic cell for a marrow-derived blood cell closely adjacent to a non-marrow-derived hepatic cell. Fig 4a shows normal male murine liver co-labeled for Y chromosome (pink) and HNF-1 (green). Nuclear DNA is stained blue by DAPI dye. Autofluorescence of hepatocyte cytoplasm and red blood cells is greenish-yellow, and yellow, respectively. Nearly all cells contain at least one Y chromosome whereas only hepatocytes and cholangiocytes express HNF-1. Figure 4b shows normal female liver. There was no Y chromosome detected in any of the cells, indicating that false positive detection of Y chromosome in transplant recipients was very unlikely. Nuclear labeling of HNF-1 is identical to that of the male liver, as expected. Figure 4c and d shows a marrow-derived hepatocyte in the liver of a mouse from the experimental group receiving no liver-specific irradiation. This photograph was taken from an animal sacrificed 1 week after withdrawal of the DDC diet. Figure 4d is an enlargement of 4c. Note the cytoplasm of the hepatocyte can be seen completely encircling the nucleus, and that the Y chromosome and HNF-1 labeling

are confined to the nucleus. For further assurance that this was not merely a blood cell overlying, or very closely adjacent to, a hepatocyte, the slide was then imaged by confocal microscopy (Figure 5). The co-labeling of Y and HNF-1 are confirmed to be contained within the same nucleus, rather than an artifact of proximity or overlap.

Double-labeled hepatocytes, such as the one in Figure 4, were sought, field by field, in tissue sections from each of our mice. In preliminary counts, out of approximately 37,500 hepatocytes counted from mice in the experimental groups, 4 marrow-derived hepatocytes were found at 1, 9, and 12 weeks after DDC treatment. 3 of the 4 hepatocytes found were from the experimental group not receiving liver-specific irradiation. From 5,100 hepatocytes counted from mice in the control group, 1 marrow-derived hepatocyte was observed. Marrow-derived cholangiocytes or oval cells were not found. These results are summarized in Table 1.

The rarity of the marrow-derived hepatocytes limited our ability to draw a temporal relation between recovery from toxic injury and the appearance of these cells. We were also unable to determine whether or not the toxic damage from DDC, with or without irradiation, caused an increase in the number of marrow-derived hepatocytes from baseline because so few were found.

In attempting to count marrow derived hepatocytes it became apparent that there were potential pitfalls to this process. First, as the injury model had induced an inflammatory response, there were a large number of inflammatory cells in the liver tissue. These cells were in very close contact with many of the hepatocytes, and it could be difficult to determine whether a nucleus containing a Y chromosome was from a blood cell or a hepatocyte. In some cases, confocal microscopy would be necessary. Figure 6 is highly

suggestive of fusion between a male marrow-derived (likely inflammatory) cell and a female recipient hepatocyte. Here the hepatocyte appears binucleate, as normal hepatocytes may be. However, HNF-1 staining is absent from the y-labeled nucleus, and the HNF-1-positive nucleus is Y chromosome-negative. These images clearly demonstrate the critical importance of using a nuclear marker of hepatocyte phenotype in these experiments.

E. Discussion:

Our studies did not show that bone marrow cells could become oval cells. Since the source of oval cells in the liver has not yet been determined unambiguously, and since bone marrow stem cells have been shown to display some of the same cell markers as oval cells (35, 36), it is not unreasonable to hypothesize that progenitor cells of marrow origin could act as a source of oval cells in the liver. Further research needs to be done to elucidate a possible link between these two cell populations.

Our studies did show that, as in previous studies, murine bone marrow cells are able to engraft in the liver and become hepatocytes after sex-mismatched bone marrow transplant with or without subsequent liver damage. We observed co-labeling of the Y chromosome and HNF-1 in the single nucleus of single hepatocyte cells.

In the past, recipient phenotype has been identified using immunohistochemical or immunofluorescent detection of cytoskeletal proteins such as cytokeratin or secreted proteins such as albumin. Both of these techniques can lead to incorrect interpretation of cells since their labeling location is in the periphery of the cell where borders between two cells may not be readily apparent. Our findings of hematopoietic cells closely adjacent to liver cells make it clear that when employing a co-labeling technique for identification of cells of both donor and recipient origin, it is essential to use markers located in the nucleus of the target cell. This approach reduces the chance of mistaking a marrow derived hepatic cell for a marrow-derived blood cell closely adjacent to a non-marrow-derived hepatic cell. Our studies show that HNF-1 is an appropriate nuclear marker for recipient phenotype in this model. Due to the specificity and location of HNF-1 labeling, it proves to be a reliable nuclear marker of

liver hepatocyte and cholangiocyte phenotype. In addition, immunofluorescent localization of HNF-1 is compatible with Y-FISH in paraffin sections.

Our use of Y chromosome detection by FISH was also a good choice for its specificity for donor phenotype and for its nuclear labeling location. This is a more reliable technique than previously employed methods because it is not subject to the potential unreliability of reporter transgene expression, as can occur with LacZ or green fluorescent protein (GFP). One pitfall to the use of the Y chromosome in paraffin sections, though, is that the Y chromosome takes up only a small portion of the nucleus, while the hepatocyte nucleus itself is relatively large. Therefore since we are examining the tissue in one plane of section only, there is a chance that we are not in the same plane of section that the Y chromosome is in, and as a result we may underestimate the number of marrow-derived hepatocytes in a given tissue. In male controls, approximately 80% of hepatocytes are Y-positive when using FISH labeling on 3 micrometer sections, so the magnitude of the false negative error is on the order of 20%.

Further analysis of our specimens via three-dimensional imaging with confocal microscopy allowed us to unequivocally ensure that the Y chromosome and HNF-1 labeling were indeed in the same nucleus and not merely artifact arising from overlap of a blood cell above or below a hepatocyte.

Even with the use of nuclear labels and confocal microscopy, the possibility of a single nucleated cell as the result of nuclear as well as cytoplasmic fusion still remains a possibility. In the highly fusogenic environment of an inflammatory reaction, it is likely that fusion indeed occurs between donor-derived inflammatory cells and recipient hepatocytes. Inflammation is induced both in injury studies as well as in studies that do not intentionally

create specific injury, as the whole body irradiation required for obliteration of the recipients own bone marrow will create some degree of inflammation. Our study was not specifically designed to detect fusion events, and the rarity of marrow-derived hepatocytes in this model limits its utility in addressing the fusion issue. The phenomena of fusion and plasticity are not mutually exclusive. To unequivocally distinguish marrow-derived hepatic cells arising from differentiation versus cell-cell fusion, a different model, such as the FAH mouse model of tyrosinemia, would be required (65, 81). Since fusion, inflammatory cell infiltration, and location of labeling in the cell can all affect the counting of putative marrow-derived hepatocytes, these factors are likely a significant cause of discrepancy in various authors' quantitative reports on the frequency with which marrow-derived hepatocytes are found.

We employed an injury model to inhibit intrahepatic progenitors including oval cells and hepatocytes, and therefore encourage the conversion of marrow-derived cells into hepatic cells. We found marrow-derived hepatocytes both in animals receiving damage, but also in control animals receiving no post-myeloablative damage. In addition, we did not find a difference between animals receiving DDC and animals receiving DDC plus liver-specific irradiation. While these results would imply that damage does not play a role in the phenomenon of bone marrow to hepatocyte conversion, this is not a conclusion that can be definitively drawn from this work since there were a very low number of marrow-derived hepatocytes found overall.

While many questions remain to be answered when it comes to the hematopoietic stem cell and its potential for plasticity, the approach of using adult stem cells in cellular therapy, regeneration medicine, and in the treatment of tissue injury or disease remains an attractive one in many ways. The moral and ethical barriers that limit use of human

embryonic stem cell experimentation and use are much less of a concern with adult stem cells. Their easy accessibility in the circulation and bone marrow make hematopoietic stem cells attractive for use. Harvesting of these cells for use in therapies would be relatively easy and minimally invasive, and therefore not limited by the shortage problems encountered with organ transplantation. One can imagine a strategy in which bone marrow could be extracted from a patient, modified, and given back as autologous cell/gene therapy for the treatment of many diseases.

While adult stem cells show potential promise for clinical use in hepatology, much more must be understood about these cells before they can be translated into clinical use. We need to better understand the driving forces behind engraftment of marrow-derived hepatic cells, specifically, their role in both physiological maintenance and in liver injury. Understanding the mechanism of transdifferentiation or fusion events could allow us to deliver corrective genes in cell therapy and regenerative medicine. In the liver, it is also becoming clear that marrow-derived cells directly influence the deposition of fibrotic matrix proteins by stellate cells, independent of either fusion or transdifferentiation. Thus, it will be critical to define the role of marrow-derived cells in the progression from inflammation to fibrosis and cirrhosis in order to identify potential targets for therapeutic intervention.

The adult stem cell is potentially a powerful source of clinically useful treatment for various diseases. Improvement of our understanding of these cells may revolutionize how we treat tissue injury and disease. All medical disciplines stand to benefit greatly from the potential uses of these cells.

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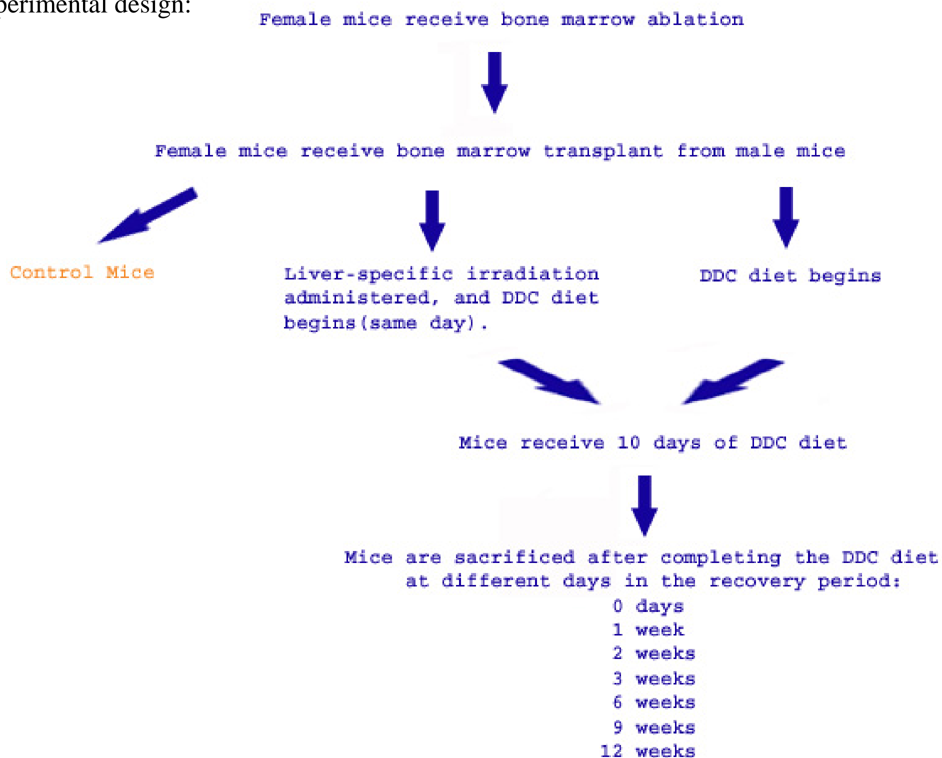
a. Experimental design:



b. Liver Irradiation Apparatus

Figure 1. a. A flowchart of the experiment design. b. Liver specific irradiation apparatus. The radiation source was placed in the ring at the top. The mouse was anesthetized and placed in a holding box. A lead shield limited irradiation to the upper abdomen.

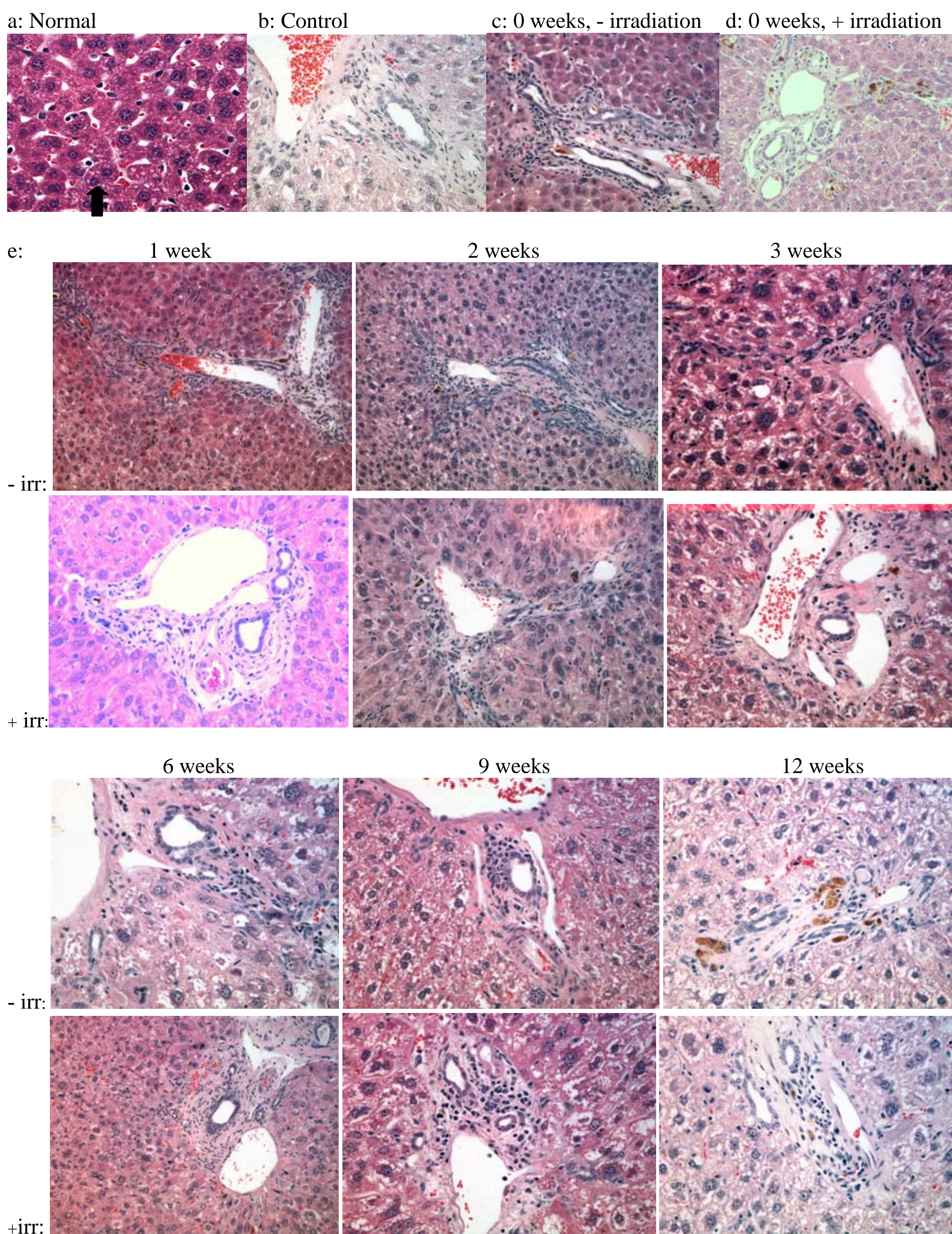


Fig 2: Hematoxylin and Eosin staining of murine liver sections. a: Normal. b: Control group which received bone marrow transplant without liver specific irradiation or DDC. No apparent abnormalities are seen in a or b. Note the presence of multi-nucleated cells in normal liver (arrow in 2.a.). c: Liver at 0 weeks after DDC treatment without liver specific irradiation. d: Liver 0 weeks after DDC treatment in addition to liver specific irradiation. e: Comparison of liver sections from animals without liver specific irradiation (- irr) and animals with liver-specific irradiation (+ irr) at different time points after completion of DDC diet (1, 2, 3, 6, 9, 12 weeks after DDC).

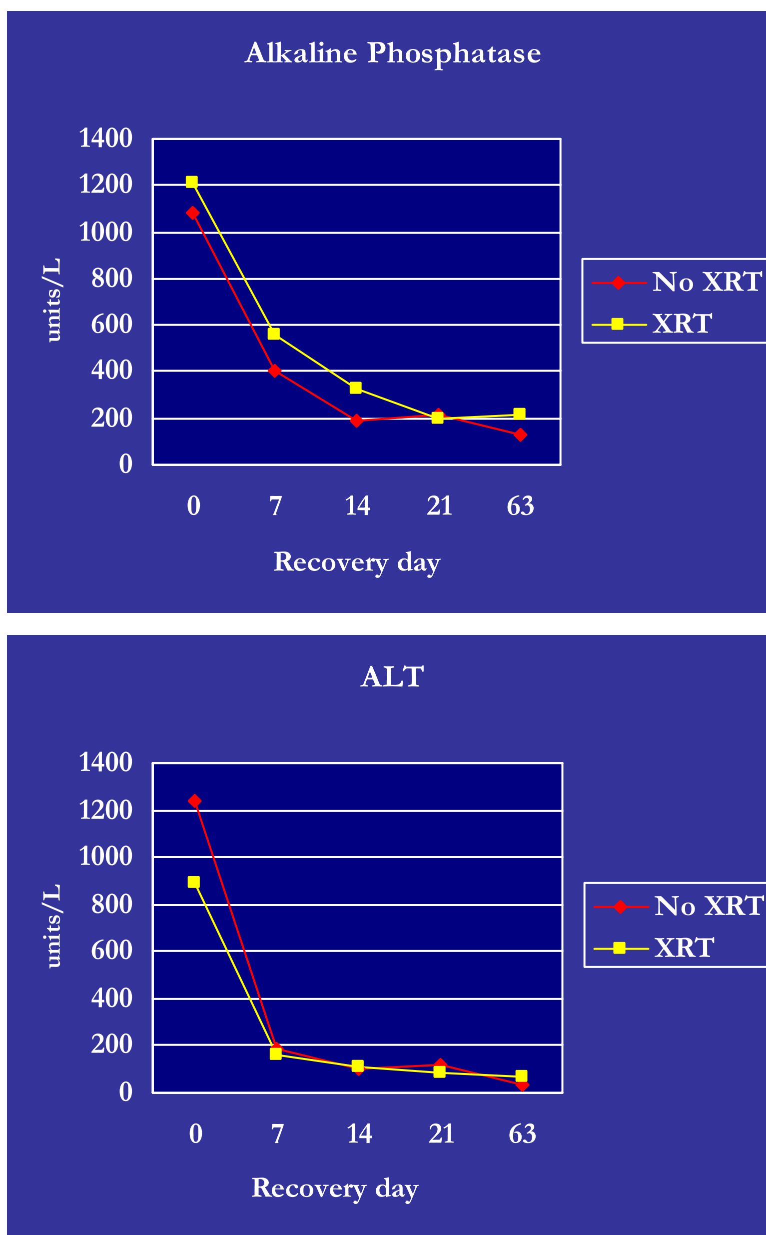


Figure 3. DDC-induced biochemical injury is reversible and unaffected by hepatic irradiation with 1000 rads. ALT= alanine transaminase. XRT= x-ray therapy (liver-specific irradiation).

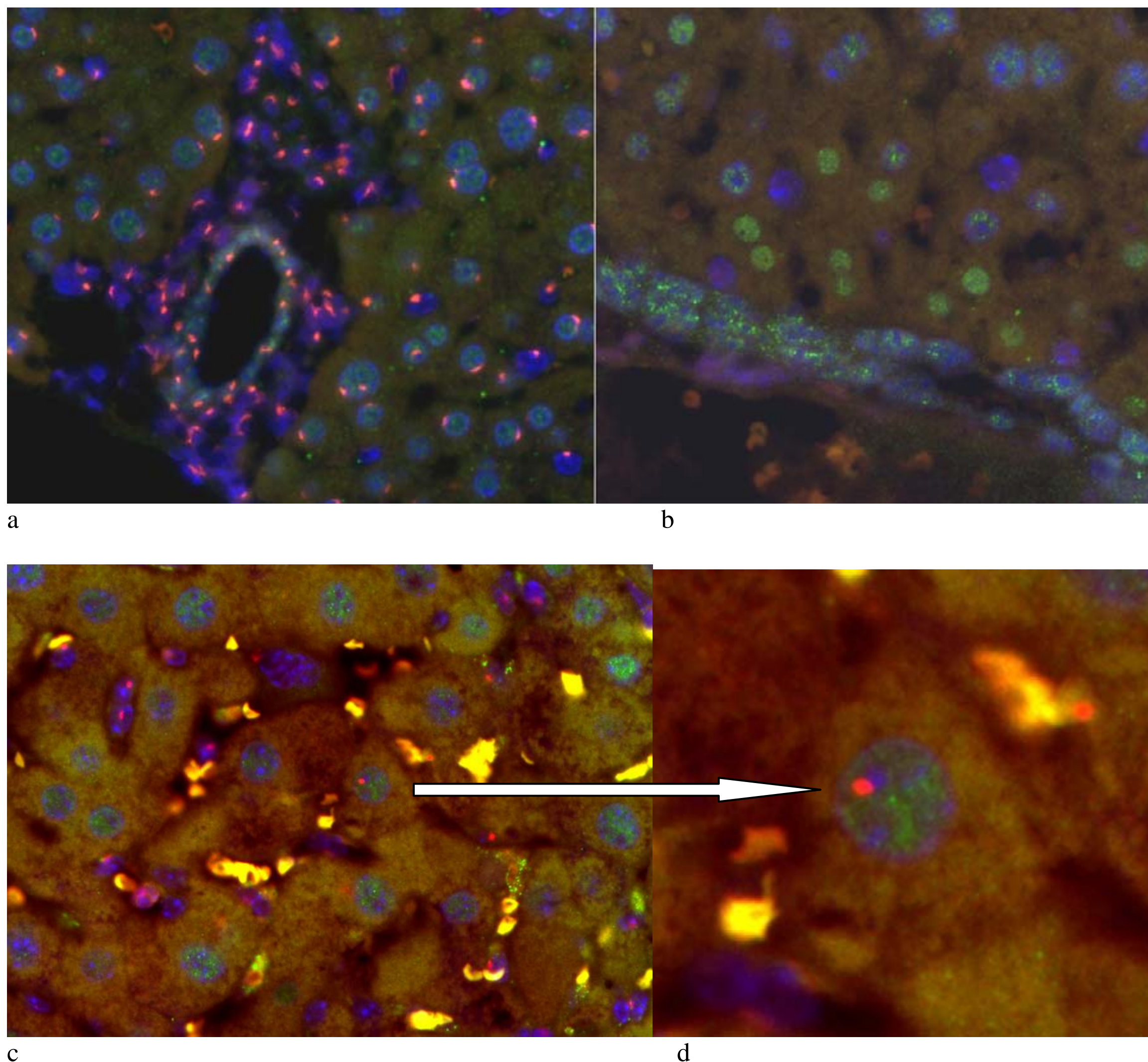


Figure 4. FISH and Immunofluorescence co-localization of Y chromosome and HNF-1 in liver. Y chromosome is pink, HNF-1 is green. Nuclear DNA is stained blue. Autofluorescence of the hepatocyte cytoplasm is green-yellow. Red blood cell autofluorescence appears bright yellow. Both the Y chromosome and HNF-1 labeling are found in the nucleus. Figure 4a shows normal male liver. Most hepatocytes contain at least one Y chromosome. Hepatocytes and cholangiocytes express HNF-1. Figure 4b shows the complete absence of Y chromosome staining in normal female liver. Nuclear labeling of HNF-1 is identical to that of the male liver. Figure 4c shows a marrow-derived hepatocyte in a mouse from the experimental group receiving no liver-specific irradiation. This animal was sacrificed 1 week post DDC insult. Figure 4d is an enlarged version of the 4c. It shows clearly the nucleus co-stained for Y-chromosome and HNF-1 as well as the hepatocyte cytoplasm completely encircling the nucleus.

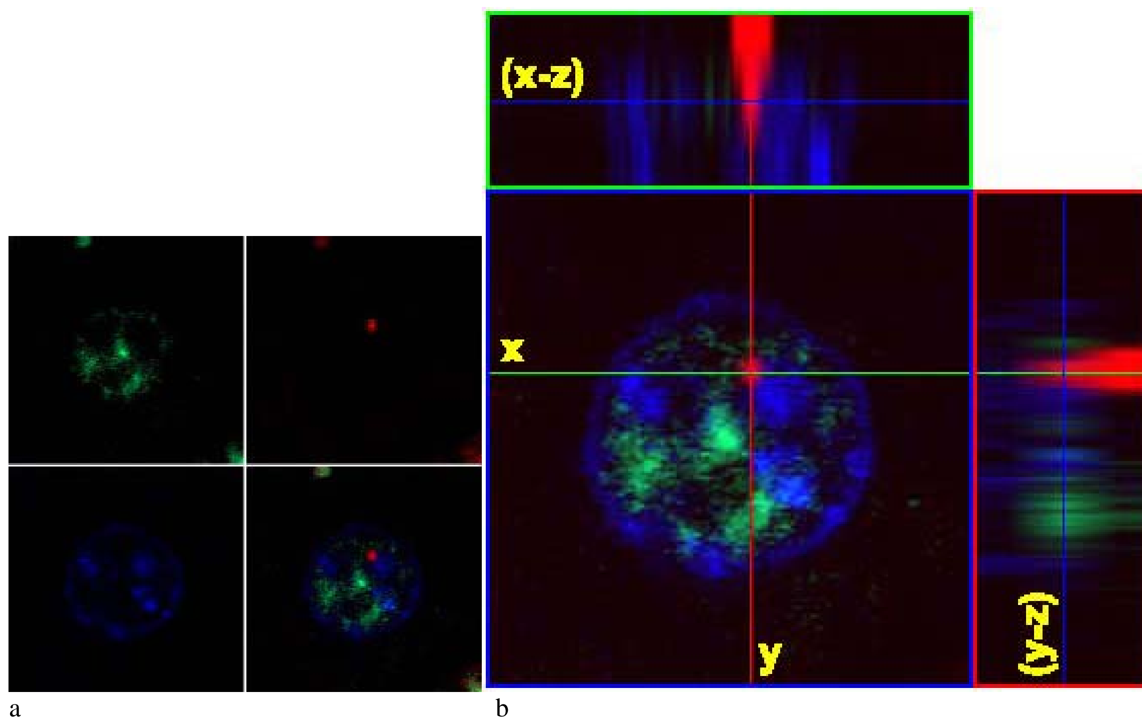


Figure 5. Confocal microscopy of Y and HNF-1 co-labelled hepatocyte. With confocal microscopy, the co-labeling is confirmed to be contained within the same nucleus without any other cells intruding on this space. Y-chromosome is pink, HNF-1 is green. Nuclear DNA is stained blue. Figure 5a shows each labeling separately, and in the lower right, the total overlay. Figure 5b shows the y-chromosome and HNF-1 labeling in both the x-z and y-z planes of imaging, and therefore are located in the same nucleus.

Table 1: Y-Positive, HNF-1 Positive, Liver cells

	Number Y-positive, HNF-1 positive cells counted :	Weeks after DDC recovery:	Total HNF-1 positive cells counted:
Control:	1	N/A	5,100
Subtotal:	1		5,100
Experimental:			37,500
- irradiation	1	1 week	
- irradiation	1	9 weeks	
- irradiation	1	12 weeks	
+ irradiation	1	12 weeks	
Subtototal:	4		37,500
Total:	5		85,200

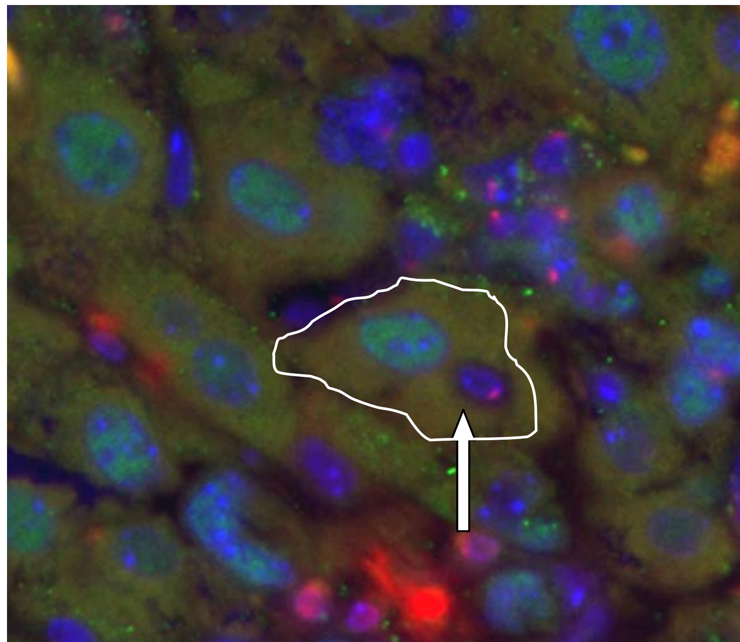


Figure 6. Pitfalls in the search for a marrow-derived hepatocyte. Y-chromosome is pink, HNF-1 is green. Nuclear DNA is stained blue. A potential marrow-derived hepatocyte is circled in white. The cell resembles a binucleate hepatocyte, one nucleus (white arrow) is not HNF-1 positive.