Correction of copper metabolism is not sustained long term in Wilson's disease mice post bone marrow transplantation

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Abstract

Purpose Alternative cell sources have been sought for the treatment of liver diseases, since liver cells are in short supply for cell transplantation. Although bone marrow-derived cells have been investigated as an alternative cell source, few studies have demonstrated long-term disease correction. Here we examined bone marrow stem cell transplantation into the toxic milk (*tx*) mouse model for Wilson's disease, a mild liver disease characterized by hepatic copper accumulation. The aim of this study was to determine whether bone marrow cells engrafted in the liver could sustain correction of abnormal copper metabolism in the *tx* mouse.

Methods Bone marrow cells were isolated from congenic normal mice, transduced to express enhanced green fluorescent protein, sorted for stem cell (Sca-1) and lineage cell (Lin) surface markers, and then transplanted into sublethally irradiated normal or *tx* mice. Analysis for donor cell activity and distribution was undertaken 5 and 9 months post-transplant to allow for sufficient time to repopulate the liver and demonstrate disease correction.

Results Donor bone marrow cells engrafted in both normal and *tx* mouse liver within 5 months. Significantly

reduced liver copper was found in *tx* mice with donor cell liver engraftment at 5 months post-transplant compared to controls, demonstrating partial correction of abnormal copper metabolism in the short term. However, disease correction was not maintained 9 months post-transplantation. Lin[−]Sca-1⁺ cells were more likely to partially correct disease than Lin⁺Sca-1[−] cells in the short term.

Conclusion These results demonstrate that bone marrow transplants cannot maintain disease correction in a mouse model of mild hepatic damage, although initial copper metabolism correction was observed.

Keywords Wilson's disease · Copper · Bone marrow stem cell · Liver · Toxic milk mouse

Introduction

A readily available source of cells for patients with liver disease may help alleviate the worldwide shortage of livers for transplantation, since liver cell transplant programs are in direct competition with whole organ programs for this scarce resource. Bone marrow (BM) provides a more readily available source of cells and we have shown previously that they can effect disease correction in the *tx* mouse over the short term [1].

Several investigations have suggested that pluripotent stem cells residing in BM contribute to liver regeneration following liver damage [2–6]. In these studies, in mice and humans, a proportion of the regenerated hepatic cells were found to be donor-derived, demonstrating the existence of a stem cell associated with BM. Although these cells were shown to be derived from donor BM and appeared to exhibit characteristics consistent with hepatocytes, there is evidence that in many cases cell fusion between donor

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bone marrow-derived cells and recipient hepatocytes is responsible for the presence of donor cells in the liver [7], although other mechanisms have also been postulated. Whether bone marrow-derived cell therapy can sustain metabolic correction over the long term is unknown. The most important marker of clinical usefulness for cell transplants is the ability to effect liver disease correction [8].

The *tx* mouse is an animal model of Wilson's disease—an inherited disorder of the ATP7B copper transporter, resulting in high levels of hepatic copper [9]. The mouse has an equivalent point mutation in the *WND* gene to humans, causing a mild liver disease of copper overload [10–12]. Until our own recent results using the toxic milk mouse (*tx* mouse) [1], the only mouse model of metabolic liver disease to demonstrate liver disease correction following BM transplantation was the fumarylacetoacetate hydrolase (*FAH*^{−/−}) knockout mouse [4]. Without treatment of the metabolic derangement, liver disease in the *FAH* mouse model is lethal. The liver environment in this particular model therefore exerts a significant advantage (strong selective pressure) toward donor cells that are metabolically normal. By contrast, the *tx* mouse model has mild liver disease and therefore only a mild selective repopulating pressure is present.

As hepatocyte replacement by BM cells is a slow and rare event under physiologic conditions [13], we hypothesized that a long time-line experiment would be required for a liver disease environment with a low selective pressure such as in the *tx* mouse. Long-term monitoring of metabolic correction in this mouse model of Wilson's disease is important to ensure that the effect is sustained. This study used fluorescent tagged and sorted donor BM cells to determine whether a subpopulation of BM could sustain disease-correctional activity within the liver over time.

Materials and methods

All reagents used were of analytical grade. Enzymes were purchased from Roche (Basel, Switzerland); antibodies were from BD Biosciences Pharmingen (San Jose, CA) and cytokines from Chemicon International (Temecula, CA) unless otherwise specified. The Royal Children's Hospital, Melbourne, Animal Research Ethics Committee approved the experimental protocol.

Mouse husbandry

All mice used in this study were kept and cared for in the mouse house facility at the Murdoch Childrens Research

Institute (Melbourne, Australia). Drinking water had an average copper content of 0.05 mg/l, whilst the normal diet (no additional added copper) of irradiated mouse pellet food (Barastoc Stockfeeds Pty Ltd, Pakenham, Vic, Australia) contained an average copper content of 0.02 mg/g (approximately 0.06 mg Cu/day). Both water and food were provided ad libitum.

Experimental mice were randomly allocated to one of twelve groups with 16 mice per group (Table 1). All groups were sub-lethally irradiated (4 Gy) the day before transplantation except for the 'non-irradiated, no transplant control' groups (Groups 11 and 12 in Table 1). At the time of transplantation, mice ranged in age from 3–4 months and were culled 5 or 9 months after transplantation. A proportion of female mice from each group were transplanted with BM cells from male donors (i.e., sex-mismatched transplants).

Bone marrow cell extraction

Ten to twelve-week-old congenic (DL) mice [10] were humanely culled for BM extraction. Femur and tibia were flushed with RPMI 1640 media containing 10% (v/v) fetal bovine serum (referred hereafter as RPMI) to extract BM cells. The cell suspension was filtered through nylon-mesh and centrifuged for 7 min (200g) at room temperature. Erythrocytes were lysed by the addition of ammonium chloride (0.83% w/v) and sodium carbonate (0.084% w/v) to the cell pellet, with tubes placed on ice for 5 min. The cell suspension was centrifuged for 5 min (100g at 4°C), after which the supernatant was discarded and the remaining cell pellet resuspended in RPMI.

Table 1 Summary of mouse transplantation groups

Group	Transplant	Donor	Recipient
1	EGFP ⁺ bone marrow (BM) cells	DL	<i>tx</i>
2	EGFP ⁺ Lin [−] Sca-1 ⁺ BM cells	DL	<i>tx</i>
3	EGFP ⁺ Lin ⁺ Sca-1 [−] BM cells	DL	<i>tx</i>
4	EGFP ⁺ BM cells	DL	DL
5	EGFP ⁺ Lin [−] Sca-1 ⁺ BM cells	DL	DL
6	EGFP ⁺ Lin ⁺ Sca-1 [−] BM cells	DL	DL
7	EGFP ⁺ BM cells	<i>tx</i>	<i>tx</i>
8	Unsorted BM cells	DL	<i>tx</i>
9	Irradiated, sham transplanted control		DL
10	Irradiated, sham transplanted control		<i>tx</i>
11	Non-irradiated, no transplant control		DL
12	Non-irradiated, no transplant control		<i>tx</i>

Lineage depletion

Dynabeads (M-450) coated with sheep anti-Rat IgG (Dynal, Oslo, Norway) were used to lineage-deplete BM cells following incubation with rat monoclonal antibodies directed against surface markers of T lymphocytes (anti-mouse CD3), B lymphocytes (anti-mouse 45R, B220), granulocytes/neutrophils (anti-mouse Ly-6), monocytes/macrophages (anti-mouse macrophage F4/80), and erythrocytes (anti-mouse TER-119, Ly-76).

Transduction and flow cytometry of bone marrow cells

A congenic mouse expressing enhanced green fluorescent protein (EGFP) is not available; therefore BM cells were retrovirally transduced to express EGFP. The LuvGM retroviral vector [14] containing the EGFP gene was transduced into the GP + E-86 ecotropic retroviral producer cell line. The transduced retroviral producer cell line was free of helper-virus and had a titer of 3.87×10^6 infectious units/ml. Transduction of mouse BM cells was performed by co-cultivation of mouse cells on irradiated (40 Gy) retroviral producer cells, polybrene (8 μ g/ml), and a cytokine mix. This recombinant cytokine mix contained: mouse interleukin-3 (IL-3) 6 ng/ml; human interleukin 6 (IL-6) 10 ng/ml; and murine stem cell factor (SCF) 50 ng/ml. After a 3-day incubation period (37°C, 5% CO₂), the BM cells were collected and sorted by flow cytometry using a FACStar flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). The BM cells were sorted for EGFP and stem cell antigen-1 (Sca-1) expression. Sorting for the stem cell antigen c-kit was not performed due to low cell numbers. Routine analysis of cell populations and post-transplant analysis of BM, spleen, and blood was completed using a LSRII flow cytometer and fluorescent labeled Sca-1 antibody (Becton-Dickinson, Franklin Lakes, NJ).

Separation of the different cell populations for transplantation

Four different categories of BM cells were transplanted into the recipient mice (Table 1). The first category is made up of BM cells which had been extracted, retrovirally transduced to express EGFP then sorted by flow cytometry to contain only cells expressing EGFP (i.e., EGFP⁺ BM cells; groups 1, 4, and 7 in Tables 1 and 2). The second category contained sorted BM stem cells, which had been extracted, lineage depleted, retrovirally transduced and then cytometrically sorted for EGFP expression and Sca-1 expression (i.e., EGFP⁺Lin⁻Sca-1⁺ BM cells; groups 2 and 5 in Tables 1 and 2). The third category contained sorted “non-stem” BM cells

which had been extracted, retrovirally transduced, and then cytometrically sorted for EGFP expression and lack of Sca-1 expression (i.e., EGFP⁺Lin⁺Sca-1⁻ BM cells; groups 3 and 6 in Tables 1 and 2). The fourth and final category contained BM cells which had been extracted, but had not been transduced or flow cytometrically sorted (i.e., unsorted BM cells; group 8 in Tables 1 and 2).

Transplantation of bone marrow cells

For intravenous (I.V.) transplantation, a suspension of BM cells (100 μ l) was injected into the mouse tail vein. The low cell yield after cell sorting limited the transplant dosage of EGFP-expressing BM cells per mouse to 5×10^5 cells. Therefore, Groups 1, 4, and 7 were transplanted with 5×10^5 EGFP⁺ BM cells (Tables 1 and 2). Approximately 30% of these EGFP⁺ cells were Sca-1⁺ after the 3-day retroviral transduction, therefore to correlate dosages between the different populations, adjusted numbers of cells were transplanted as follows: Groups 2 and 5 were transplanted with 1.5×10^5 EGFP and Sca-1-expressing, lineage-negative (EGFP⁺Lin⁻Sca-1⁺) BM cells; Groups 3 and 6 were transplanted with 3.5×10^5 EGFP expressing, lineage positive, Sca-1 negative (EGFP⁺Lin⁺Sca-1⁻) BM cells. Group 8 was transplanted with 5×10^6 whole BM cells. To evaluate the effects of sublethal irradiation on the parameters, Groups 9 and 10 comprised irradiated mice injected with RPMI as sham control groups.

Tissue collection and analysis

Tissue was collected from the liver, spleen, tail, kidney, brain, small intestine, lung, heart, and BM and stored

Table 2 Engraftment of liver and BM of recipient mice with donor BM cells at 5 and 9 months post-transplant

Group	Donor → Recipient	Cells transplanted	Liver		BM	
			5 m	9 m	5 m	9 m
1	DL → tx	EGFP ⁺	2	0 ^a	6	7 ^a
2	DL → tx	EGFP ⁺ Lin ⁻ Sca-1 ⁺	4 ^a	5	6 ^a	6
3	DL → tx	EGFP ⁺ Lin ⁺ Sca-1 ⁻	0	2	6	7
4	DL → DL	EGFP ⁺	3	2	5	8
5	DL → DL	EGFP ⁺ Lin ⁻ Sca-1 ⁺	1	0 ^a	4	2 ^a
6	DL → DL	EGFP ⁺ Lin ⁺ Sca-1 ⁻	0	0	4	1
7	tx → tx	EGFP ⁺	0	0	3	1
8	DL → tx	Unsorted BM cells	5	2 ^b	7	5 ^b

Data presented as number of animals per group with liver or BM repopulation ($n = 8$ for each group)

^a Only 7 mice in these groups

^b Only 6 mice in these groups

immediately at -80°C for DNA and RNA analysis. Additional liver tissue was collected from all experimental animals, and stored in acid-washed tubes at -20°C for trace metal analysis. Levels of copper were determined by atomic absorption spectroscopy (AAS) using the method of Michalczyk et al. [15]. Briefly, the tissues were dehydrated in an 85°C oven for 1 week, then dissolved in 0.5 ml of 65% suprapure nitric acid (Merck, Germany) at room temperature for 1 h and then at 65°C for a further 3 h. The samples were then diluted with 3 ml deionized water for analysis on a Varian SPECTRAA-800 instrument in the flame mode. All results were expressed as $\mu\text{g/g}$ dry weight of tissue. Blood plasma was also collected for analysis of ceruloplasmin oxidase activity, as determined using the method of Schosinsky et al. [16].

Polymerase chain reaction of *mWND* gene

Genotyping of the tissues was performed using the polymerase chain reaction (PCR) to amplify the mouse Wilson's disease gene (*mWND*). The product was then restriction enzyme digested with *NcoI* enzyme [17] to identify the genotype. A PCR product of 659 bp, containing the gene sequence for a normal DL mouse, produced four fragments of 345, 193, 103, and 18 bp in length after *NcoI* digestion. The *tx* mouse mutation removed a *NcoI* digestion site, resulting in only three fragments 448, 193, and 18 bp. Donor cell repopulation was also analyzed using PCR amplification of the following genes: EGFP [18]; sex-determining region Y (Sry) for male to female transplants [19]; and myogenin (myog) gene used as a housekeeping positive control [19].

Reverse transcription-PCR of ATP7B and EGFP mRNA

RNA was isolated from samples using the TRI[®] reagent (Molecular Research Center, Cincinnati, OH) method. The mRNA was reverse transcribed and a nested PCR protocol used to amplify ATP7B, the copper transporter product of the Wilson's disease gene [17]. PCR of cDNA resulted in a 653 bp product that, when digested with *NcoI*, produced two fragments of 417 and 236 bp for the *tx* mouse mutation, and three fragments (285, 236, and 132 bp) for the congenic DL mouse. EGFP mRNA was analyzed by PCR amplification [18].

Data analysis

Analysis of data by an independent-samples *t*-test was performed using the SPSS (Statistical Package for Social

Sciences—Version 12) program. Data was considered significant when $P < 0.05$. All data are presented as mean $\pm$ SE. Data points more than two standard deviations away from the mean were considered outliers and removed.

Results

EGFP and Sca-1 expression

EGFP mRNA and protein were expressed via the retroviral LTR promoter in cells of the liver, BM, spleen, and blood of recipient mice within 5 months post-transplantation, as determined by RT-PCR or flow cytometry.

Flow cytometric analysis of BM, spleen and blood cells in recipient mice showed expression of EGFP between 0.1% and 9.4% in BM (average of $1.7 \pm 0.7\%$, $n = 18$), 0.1–10.9% in spleen (average $1.7 \pm 0.5\%$, $n = 33$) and 0.1–10.7% in blood (average $2.7 \pm 0.9\%$, $n = 15$). Downregulation of the retroviral promoter appeared to occur by 9 months post-transplantation since EGFP DNA could be detected in 25 of 32 recipient mice without measurable fluorescent protein using flow cytometry (data not shown).

The average percent of Sca-1 positive cells by flow cytometry in BM was $5.0 \pm 0.2\%$ $n = 185$ spleen was $28.2 \pm 1.0\%$ $n = 122$ and blood was $5.5 \pm 0.6\%$ $n = 112$. The percentage of BM cells in recipient mice that were positive for Sca-1 was not influenced by age, sex, cells transplanted, irradiation or strain of mouse.

Repopulation

Bone marrow engraftment was identified in over 60% (78/123) of the mice exposed to sub-lethal irradiation (Table 2). Engraftment was defined by the presence of donor DNA in recipient tissue as characterized by PCR for the Wilson's disease gene (*mWND*), EGFP or Sry DNA (Figs. 1–3). Liver engraftment with donor BM cells was found in many of the transplant groups (Table 2) at both the 5- and 9-month time points. Liver ATP7B (Wilson's disease gene product) mRNA was below levels of detection in all 21 *tx* mice that showed presence of DL donor cells in liver at either 5 or 9 months by DNA analysis.

A comparison of the distribution of donated cells showed that all mice with liver engraftment also had spleen repopulation and some ($n = 4/26$) had small intestine repopulation, whilst none had engraftment in the lung, heart, kidney, or brain.

Liver copper concentration

As there was no significant difference in liver copper concentrations between liver engrafted mice in Groups 1–3, these groups were combined (Fig. 4). Transplanted BM cells significantly reduced liver copper content in recipients demonstrating partial correction by donor cells, but only at 5 months. Despite the presence of donor cells at 9 months there was no statistically significant difference in liver copper concentration. *Tx* mice that were transplanted with DL cells, but in which significant donor cell engraftment could not be detected (i.e., the gray columns in Fig. 4), had a trend toward lower mean liver copper concentrations than control *tx* mice, suggesting that some animals may have had liver repopulation at levels below the detection limit of PCR.

Normal DL mouse average liver copper concentrations ($\mu\text{g/g}$ dry weight of tissue) were: 17 ± 3 for 10–12 week old mice ($n = 7$); 14 ± 1 for 8–9 m old mice ($n = 16$); and 12 ± 2 for 12–13 m old mice ($n = 16$). For control *tx* mice the average liver copper concentration ($\mu\text{g/g}$ dry weight of tissue) were: 669 ± 57 for 10–12 week old mice ($n = 7$); 694 ± 41 for 8–9 m old mice ($n = 15$); and 732 ± 113 for 12–13 m old mice ($n = 13$).

The *tx* to *tx* transplant group (Group 7) had no evidence of liver engraftment (Table 2) or improvement in liver copper compared to controls (708 ± 37 , $n = 8$ and 608 ± 77 , $n = 8$, at 5 and 9 months post-transplant respectively). Although the whole BM transplant group (Group 8) (DL to *tx* transplantation) contained several mice with liver repopulation (Table 2), the donor cells did not aid in liver disease correction as significantly as seen with EGFP-transduced cells (647 ± 86 , $n = 3$ and 622 ± 101 , $n = 3$, at 5 and 9 months post-transplant respectively).

Fig. 1 PCR/*Nco*I digest profile of the *mWND* gene from BM stem cell transplanted *tx* mice. This figure demonstrates successful DL mouse donor cell engraftment in the liver, spleen and BM of a *tx* mouse. Lane 1: DL mouse liver; lane 2: 10% DL DNA mixed with *tx* DNA; lane 3: *tx* mouse liver; lanes 4–6: *tx* mouse transplanted with BM stem cells showing donor cell engraftment in liver, spleen and BM. The tail DNA digest in lane 7 confirms that the recipient was a *tx* mouse

Fig. 2 PCR of the EGFP gene from a BM stem cell transplanted mouse. This figure demonstrates successful donor cell engraftment in liver, spleen and BM. Lane 1: EGFP positive DNA; lane 2: Mouse DNA with no EGFP; lanes 3–5: Mouse transplanted with BM stem cells showing donor cell repopulation in liver, spleen and BM. The tail DNA in lane 6 confirms that the recipient mouse was EGFP negative. Myog was used as a positive control

Fig. 3 PCR of the Sry gene from a female mouse transplanted with male BM stem cells. This figure demonstrates successful donor engraftment and repopulation in liver, spleen and BM of a sex-mismatched transplant. Lane 1: Male liver; lane 2: 10% Male DNA mixed with female DNA; lane 3: Female liver; lanes 4–6: Female mouse transplanted with male BM stem cells showing donor cell engraftment in the liver, spleen and BM. Tail DNA PCR in lane 7 confirms that the transplanted mouse was female. The myog gene was used as a positive control

Ceruloplasmin oxidase activity

There was a significant increase in *tx* ceruloplasmin oxidase activity in donor cell engrafted mice after 5 months (Group 1–3) compared to controls and transplanted mice with no significant liver engraftment (Fig. 5). At 9 months post-transplant, despite the presence of donor cells, there was no statistically significant improvement in ceruloplasmin oxidase activity. There was also no significant difference in ceruloplasmin oxidase activity between ‘no transplant’ and sham-transplanted controls for each strain, irrespective of irradiation (i.e., DL Groups 9 and 11, and *tx* Groups 10 and 12) (Table 1), and so the data for these paired groups were combined. Normal DL mouse ceruloplasmin oxidase activity (IU/L) was: 21 ± 1 for 10–12 week old mice ($n = 7$); 33 ± 2 for 8–9 m old mice ($n = 16$); and 38 ± 3 for 12–13 m old mice ($n = 16$). Average ceruloplasmin oxidase activity for control *tx* mice (IU/L) was: 0.3 ± 0.1 for 10–12 week old mice ($n = 7$); 7 ± 1 for 8–9 m old mice ($n = 15$); and 12 ± 2 for 12–13 m old mice ($n = 13$).

Fig. 4 Comparison of copper concentration in control, non-engrafted and liver engrafted *tx* mice at 5 and 9 months post-transplantation. The mean copper concentration in *tx* mice transplanted with EGFP⁺ BM cells (Groups 1–3) showing liver engraftment (white columns) compared to ‘no engraftment mice (animals transplanted with BM cells, but did not exhibit liver engraftment; gray columns) and control mice (black columns), at 5 and 9 months post-transplantation. The results show that *tx* mice with liver engraftment had a significantly lower liver copper concentrations at 5 months post-transplant, whilst there was no significant difference at 9 months post-transplant, demonstrating that the transplanted BM cells are unable to maintain long-term functional capacity

Fig. 5 Comparison of ceruloplasmin oxidase activity in control, non-engrafted and liver engrafted *tx* mice at 5 and 9 months post transplantation. The mean ceruloplasmin activity in *tx* mice transplanted with EGFP⁺ BM cells (Groups 1–3) showing liver engraftment (white columns) compared to ‘no engraftment’ mice (animals transplanted with BM cells, but did not exhibit liver engraftment; gray columns) and control mice (Groups 10 and 12; black columns) 5 and 9 months post-transplantation. The results confirm that *tx* mice with liver engraftment have higher ceruloplasmin oxidase activity at 5 months post-transplant, but not significantly at 9 months post-transplant showing transplanted BM cells unable maintain disease correction long-term

As expected, there was no evidence of improvement in ceruloplasmin parameters in *tx* mice that had received *tx* BM transplants. Despite evidence of engraftment in the liver of *tx* mice that had received unsorted DL BM there was no improvement in ceruloplasmin oxidase activity.

Discussion

We have shown that BM cells transplanted into *tx* mice engraft in the liver and that these cells function to produce partial metabolic disease correction through a significant decrease in liver copper and a significant increase in ceruloplasmin oxidase activity 5 months post-transplantation. This effect was not sustained over a 9-month period post-transplant.

Why was metabolic correction not sustained? Rejection is unlikely as the donor cells and recipient mouse were congenic and donor cells were still present in a significant enough quantity to be detectable by PCR at the longer time point. Donor cells may have become metabolically exhausted and ceased functioning effectively or been overwhelmed by the toxic effect of progressive copper accumulation in the *tx* mouse. Further delineation of the cell type involved in partial disease correction in this model type as well as characterization of the mechanism involved (including the issue of cell fusion), may shed further light on this issue.

Tx mice transplanted with an enriched Sca-1⁺ BM cell population were more likely to have hepatic engraftment than other BM cell populations. The Sca-1 cell surface glycoprotein is used routinely as a marker of mouse hematopoietic stem cells (HSCs), allowing a >100-fold enrichment of these rare cells from BM of adult mice [20]. The Sca-1⁺ population of cells contains significant numbers of relatively short-lived B-lineage progenitor cells, but does not generally contain myelomonocytic progenitor cells [20]. Surprisingly, long-term BM engraftment, but not disease correction, was observed in the group transplanted with Lin⁺Sca-1⁺ cells, although we cannot exclude the possibility that an occasional Lin⁺Sca-1⁺ cell was present following sorting and therefore responsible for long-term engraftment.

Under physiological circumstances the contribution of BM cells to the healthy liver is thought to be minimal [13]. In our study, DL mice transplanted with DL BM cells were detected to have donor cells in the liver, although not to the same extent as that observed with DL to *tx* transplants. Liver disease or cell death cell signals are thought to be required to recruit BM cells to the liver. The initial sub-lethal irradiation damage of recipient mice to allow engraftment of BM may therefore have created enough damage to the liver to effect signals for bone marrow recruitment [21]. Irradiation results in hepatocyte death during mitosis and thus reduces hepatocyte proliferation in response to liver damage [22], creating the opportunity for donor cells to be recruited to assist in liver repair. Without irradiation, we found no engraftment of DL BM cells in the liver of *tx* mice [1], suggesting that potential liver damage from irradiation is a prior requirement for liver engraftment.

A small number of mice that had liver engraftment also had small intestine repopulation. In human BM transplant recipients, it has been suggested that donor-derived epithelial cells substantially repopulate the gastrointestinal tract after graft-versus-host disease or ulcer formation [23, 24]. This finding supports previous studies that have shown donor bone marrow-derived engraftment in the intestine of mice after whole body irradiation (12 Gy) and transplantation with HSC (c-kit⁺Lin⁺Sca-1⁺) [25].

Although we did not expect disease correction with *tx* to *tx* transplants, we were surprised that no liver engraftment was observed. This finding suggests a defect within the hematopoietic compartment of *tx* mice whereby the donor cells have no selective advantage over endogenous BM cells. Such a defect is supported by the significantly decreased BM repopulation in *tx* BM to *tx* mouse transplant group (Group 7) compared to DL BM to *tx* mouse transplant group (Group 1). Some Wilson's disease patients have unusually low levels of circulating blood platelets (thrombocytopenia) suggesting BM dysfunction [26].

The transplantation of female mice with male donor cells (sex mismatching) resulted in no detectable difference in the engraftment potential of the cells. The sex mismatching was, however, valuable in confirming the engraftment results by detecting Sry via PCR.

In conclusion, BM cells transplanted into mice with mild metabolic liver disease are able to repopulate the liver and reduce disease in the medium, but not longer term. These results have interesting implications for liver disease in which there is not a strong selective growth advantage and suggests that liver engraftment can occur in an environment of slow cell turnover, but not in a sustained way. Further research in this area is required to define the mechanism of exhaustion of metabolic capacity post-transplantation.

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