American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
editorial@hematology.org

Donor-Cell Engineering with GSK3 Inhibitor-Loaded Nanoparticles Enhances Engraftment Following in Utero Transplantation

Tracking no: BLD-2019-001037R1-FINALFILES

Stavros Loukogeorgakis (The Children's Hospital of Philadelphia, United States) Camila Fachin (The Children's Hospital of Philadelphia, United States) André Santos Dias (The Children's Hospital of Philadelphia, United States) Haiying Li (the Children's Hospital of Philadelphia, United States) Li Tang (Massachusetts Institute of Technology, United States) Aimee Kim (University of North Carolina Hospitals, United States) Jesse Vrecenak (The Children's Hospital of Philadelphia, United States) John Stratigis (The Children's Hospital of Philadelphia, United States) Nicholas Ahn (Children's Hospital of Philadelphia, United States) Ilana Nissim (The Children's Hospital of Philadelphia,) Izhtak Nissim (University of Pennsylvania,) Antonio Moron (Federal University of São Paulo, Brazil) José Luiz Martins (Federal University of São Paulo,) William Peranteau (The Children's Hospital of Philadelphia, United States) Paolo De Coppi (UCL Institute of Child Health and Great Ormond Street Hospital, United Kingdom) Darrell Irvine (Massachusetts Institute of Technology, United States) Alan Flake (The Children's Hospital of Philadelphia, United States)

Abstract:
Host cell competition is a major barrier to engraftment following in utero hematopoietic cell transplantation (IUHCT). Here we describe a cell-engineering strategy using glycogen synthase kinase-3 (GSK3) inhibitor-loaded nanoparticles conjugated to the surface of donor hematopoietic cells in order to enhance their proliferation kinetics and ability to compete against their fetal host equivalents. With this approach, we achieved remarkable levels of stable, long-term hematopoietic engraftment for up to 24 weeks post IUHCT. We also show that the salutary effects of nanoparticle-released GSK3 inhibitor are specific to donor progenitor/stem cells, and are achieved by a pseudo-autocrine mechanism. These results establish that IUHCT of hematopoietic cells decorated with GSK3 inhibitor-loaded nanoparticles can produce therapeutic levels of long-term engraftment and could therefore allow single-step prenatal treatment of congenital hematological disorders.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: S.P.L., C.G.F., A.I.B.S.D., H.L., L.T., D.J.I., A.W.F. designed the work. S.P.L., C.G.F., A.I.B.S.D., H.L., A.G.K., J.D.V., J.D.S., N.J.A. performed the experiments and contributed to data collection. I.N. and I.N. developed the method and performed the measurements of CHIR99021. S.P.L., C.G.F., A.I.B.S.D., H.L., I.N., I.N., W.P., P.D.C., D.J.I., A.W.F. contributed to data analysis and interpretation. S.P.L., C.G.F., J.D.S., N.J.A prepared the figures. S.P.L., C.G.F., A.W.F. wrote the manuscript. S.P.L., C.G.F., A.I.B.S.D., H.L., J.D.S., N.J.A., I.N., A.F.M., J.L.M., W.P., P.D.C., D.J.I., A.W.F. contributed to the revision of the manuscript.

Non-author contributions and disclosures: Yes; We would like to thank the staff at the Metabolomic Core facility, Children's Hospital of Philadelphia for measuring CHIR99021 level in various experiments.

Agreement to Share Publication-Related Data and Data Sharing Statement: For original data, please contact sloukogeorgakis@gmail.com.

Clinical trial registration information (if any):

Donor-Cell Engineering with GSK3 Inhibitor-Loaded Nanoparticles Enhances Engraftment Following in Utero Transplantation

Short Title: Targeted GSK3 Inhibition Enhances Engraftment Post IUHCT

Authors: Stavros P Loukogeorgakis MD BSc PhD MRCS(Eng.)[1,2†], Camila G Fachin MD PhD[1,3,4†], Andre I B S Dias MD PhD[1,3,4], Haiying Li[1], Li Tang PhD[5,6], Aimee G Kim MD[1], Jesse D Vrecenak MD[1], John D Stratigis MD[1], Nicholas J Ahn MD[1], Ilana Nissim[7], Izhtak Nissim PhD[7,8], Antonio F Moron MD PhD[3], Jose L Martins MD PhD[3], William Peranteau MD[1], Paolo De Coppi MD PhD[2], Darrell J Irvine PhD[5,6,9,10,11], and Alan W Flake MD[1]

[1]Center for Fetal Research, The Children's Hospital of Philadelphia, Philadelphia, PA, USA; [2]Stem Cells and Regenerative Medicine, Institute of Child Health, University College London, London, United Kingdom; [3]Federal University of São Paulo, São Paulo, Brazil; [4]Federal University of Paraná, Curitiba, Brazil; [5]Department of Material Science and Engineering, Massachusetts Institute of Technology, Boston, MA, USA; [6]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Boston, MA, USA; [7]Division of Genetics and Metabolism, The Children's Hospital of Philadelphia, Philadelphia, PA, USA; [8]Department of Pediatrics, Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA, USA; [9]Department of Biological Engineering, Massachusetts Institute of Technology, Boston, MA, USA; [10]Ragon Institute, Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, MA, USA; and [11]Howard Hughes Medical Institute, Chevy Chase, MD, USA.

†Joint first authors: S.P.L. and C.G.F. contributed equally to this study.

Corresponding author:

Stavros P Loukogeorgakis, Stem Cells and Regenerative Medicine, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London WC1N 1EH. Tel: +44 7793058558. E-mail: sloukogeorgakis@gmail.com.

Word count for abstract: 127
Word count for text: 4,388
Figure/ table count: 6
Reference count: 53

Key points:

- Conjugation of GSK3 inhibitor-loaded nanoparticles with donor cells enhances their proliferation kinetics post in utero transplantation.
- Resulting in therapeutic levels of engraftment that could allow "single-step" in utero treatment of congenital hematological disorders.

Abstract

Host cell competition is a major barrier to engraftment following in utero hematopoietic cell transplantation (IUHCT). Here we describe a cell-engineering strategy using glycogen synthase kinase-3 (GSK3) inhibitor-loaded nanoparticles conjugated to the surface of donor hematopoietic cells in order to enhance their proliferation kinetics and ability to compete against their fetal host equivalents. With this approach, we achieved remarkable levels of stable, long-term hematopoietic engraftment for up to 24 weeks post IUHCT. We also show that the salutary effects of nanoparticle-released GSK3 inhibitor are specific to donor progenitor/stem cells, and are achieved by a pseudo-autocrine mechanism. These results establish that IUHCT of hematopoietic cells decorated with GSK3 inhibitor-loaded nanoparticles can produce therapeutic levels of long-term engraftment and could therefore allow single-step prenatal treatment of congenital hematological disorders.

Introduction

In utero hematopoietic cell transplantation (IUHCT) has the potential to cure any congenital disease that can be diagnosed prenatally and treated postnatally by hematopoietic stem cell (HSC) transplantation[1]. IUHCT is based on the pre-immune window of fetal development, resulting in donor-specific tolerance and allogeneic donor cell engraftment, without the need for myeloablation or immunosuppression[2-4]. Although stem cells from various sources might be utilized for IUHCT, the most likely candidate for broad clinical application to date is the bone marrow derived adult HSC[1].

Despite the potential advantages of IUHCT over postnatal transplantation, host cell competition remains a major barrier to overcome before IUHCT becomes a therapeutic option[1;3;4]. In the normal fetus, stem cell and progenitor populations in the liver and other hematopoietic niches have a competitive advantage over their postnatal equivalents, due to favorable cell cycling and expansion kinetics[1]. Due to this advantage, IUHCT of adult HSC yields sub therapeutic levels of long-term chimerism in many animal models with robust fetal hematopoietic compartments, including the mouse[5] and the dog[6], requiring post-natal boosting with HSC from the same donor in conjunction with non-myeloablative conditioning[7].

Glycogen synthase kinase-3 (GSK3) is a constitutively active serine-threonine kinase[8] involved in the regulation of several signaling pathways (including Wnt[9], Hedgehog[10] and Notch[11]) associated with modulation of HSC homeostasis[12-16]. The importance of GSK3 has been long suggested by the clinical finding that lithium increases circulating HSC (as CD34+ cells)[17] and peripheral blood counts[18-20] in greater than 90% of patients. In addition, there are laboratory findings that lithium increases transplantable HSC in mice[21]. Because lithium directly inhibits GSK3[22], these clinical and laboratory observations implicate GSK3 as an important regulator of HSC homeostasis[9-11;23-25].

The use of GSK3 inhibitors in experimental postnatal HSC transplantation results in enhanced repopulating capacity in vivo[26]. While the regulatory effect of GSK3 inhibitor treatment preferentially impacts the primitive hematopoietic compartment, it has been shown that this augmented reconstitution is dependent upon sustained GSK3 inhibitor administration[26]. In order to increase adult HSC competitiveness in IUHCT, the cells will need to be continuously treated with the GSK3 inhibitor. Unfortunately, fetuses are inaccessible for continuous dosing. Even if

sustained fetal treatment was possible, systemic GSK3 inhibition would affect both donor and host cells alike and may have adverse effects on fetal development.

Cell engineering with surface-conjugated synthetic nanoparticles can be a strategy for adjuvant drug delivery in cell therapies. Chemically conjugating submicron-sized drug-loaded nanoparticles directly onto the plasma membrane of donor cells enables continuous and selective pseudo-autocrine stimulation of transferred cells in vivo[27-29]. Nanoparticles have been shown to increase in vivo repopulation rates of donor HSC after HSCT in adult mice, when compared to systemic dosing[27].

We hypothesized that treatment of adult HSC with GSK3 inhibitor-loaded nanoparticles prior to IUHCT would enhance the competitive capacity of donor cells in the fetal hematopoietic environment. Here we demonstrate a remarkable increase in allogeneic donor cell engraftment after IUHCT using this strategy.

Methods

Cell isolation and animal procedures

Balb/c (H2Kd+) and B6.SJL-Ptprca-Pep3b/BoyJ (H2Kb+, CD45.1+; B6-CD45.1) mice were purchased from Jackson laboratory. C57BL/6TgN(act-EGFP)OsbY01 (H2Kb+, GFP+; B6-GFP) were kindly provided by Dr. Okabe (Osaka University). All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, and followed guidelines set forth in the NIH Guide for the NC3Rs and ARRIVE guidelines.

For the isolation of donor cells, bone marrow (BM) was harvested from 6 to 8-week-old donors (B6-GFP and B6-CD45.1) and mononuclear cells (BM-MNC) were obtained as previously described[30]. The protocol for isolation of hematopoietic stem cells (lineage-/Lin-, Sca1+, cKit+; LSK or BM-HSC) is summarized in Supplemental Methods. Intravenous IUHCT of allogenic BM-MNC (B6-GFP or B6-CD45.1; 10^7 cells) was performed at embryonic day 14 (E14) of Balb/c fetuses as previously described (Supplemental Methods)[3;30].

GSK3 inhibitor-loaded nanoparticle synthesis and conjugation on cell surface

The nanoparticles used in this study are referred to as multilamellar lipid vesicles (MLV). To synthesize MLV, we adapted the protocol described by Stephan and colleagues (Supplemental Methods and Supplemental Figure 1A)[27]. They were loaded with CHIR99021-HCl (MLV-CHIR99021), a hydrochloride of CHIR99021 that is a GSK-3α/β inhibitor with IC50 of 10nM/6.7nM in cell-free assays (Selleck Chemicals, Houston, Texas, USA). For MLV conjugation on the surface of hematopoietic cells, we mixed MLV with freshly isolated BM-MNC or BM-HSC in serum free media to achieve nanoparticle concentration of 300-900 MLV/cell and cell concentration of $6x10^6$ – $1.2x10^7$/ml. Protocols for MLV characterization are summarized in Supplemental Methods.

Experimental protocols

Protocol 1:

Following preliminary studies that assessed potential effects of MLV conjugation on the ability of BM-MNC to home to and engraft in fetal hematopoietic tissues post allogenic IUHCT (Figure 1, and Supplemental Methods), we performed a proof-of-principle in vivo study aiming to establish whether gradual release of CHIR99021 from MLV conjugated on the surface of donor (B6-GFP) BM-MNC could enhance engraftment post-IUHCT. There were three experimental groups (Figure 2A); group I: 10^7 BM-MNC without nanoparticles, group II: BM-MNC with a bolus dose of CHIR99021 similar to that loaded on MLV (BM-MNC-CHIR99021), and group III: BM-MNC cell-surface "engineered" with MLV-CHIR99021 (BM-MNC-MLV-CHIR99021).

Protocol 2:

In our next round of in vivo experiments, we sought to determine whether the GSK3 inhibitor released by MLV affects engraftment by acting on donor BM-HSC. To achieve this, we isolated LSK cells from B6-GFP BM-MNC and "decorated" their cell membrane either with empty MLV or MLV-CHIR99021. We also cultured BM-HSC over a 7-day period with (or without) a similar amount of CHI99021 to the one carried by MLV (per cell) added to culture media (Supplemental Methods). Some of the cultured cells were used for in vitro hematopoietic colony forming (CFU)

assays and real-time PCR (Wnt, Notch and Hedgehog signaling pathways; Supplemental Methods). Prior to IUHCT, "decorated" or cultured BM-HSC were mixed with fresh non-LSK BM-MNC to achieve similar proportions of BM-HSC in the 10^7 B6-GFP cells transplanted into each fetus (approximately 0,1%, or 10,000 HSC). There were five experimental groups in this study (Figure 4F); group I: BM-MNC without nanoparticles, group II: BM-MNC in which fresh BM-HSC were replaced by similar number of BM-HSC cultured without CHIR99021 [Cultured HSC (-) CHIR99021], group III: BM-MNC in which fresh BM-HSC were replaced by similar number of BM-HSC cultured with CHIR99021 [Cultured HSC (+) CHIR99021], group IV: BM-MNC in which fresh BM-HSC were "decorated" with empty MLV (Fresh HSC-MLV), and group V: BM-MNC in which MLV-CHIR99021 were conjugated on the cell surface of fresh BM-HSC (Fresh HSC-MLV-CHIR99021).

Protocol 3:

In our final in vivo experimental series, we attempted to provide more definitive evidence of targeted/pseudo-autocrine bioactivity by adapting Harrison's competitive repopulation assay[31] and using it in the setting of IUHCT. Our donor cell inoculum consisted of a one-to-one "mixture" of B6-GFP ($5x10^6$) and B6-CD45.1 ($5x10^6$) BM-MNC. MLV-CHIR99021 were conjugated either on GFP+ or CD45.1+ donor cells prior to IUHCT. There were three experimental groups in our in vivo "competitive" experiments (Figure 5A); group I: Mixture of unprocessed B6-GFP and B6-CD45.1 BM-MNC (GFP + CD45.1), group II: B6-GFP BM-MNC "decorated" with MLV-CHIR99021 mixed with unprocessed B6-CD45.1 BM-MNC (GFP-MLV-CHIR99021 + CD45.1), and group III: unprocessed B6-GFP BM-MNC mixed with B6-CD45.1 BM-MNC "decorated" with MLV-CHIR99021 (GFP + CD45.1-MLV-CHIR99021).

Outcomes

In protocols 1 and 2, fetal and postnatal survival (up to 6 months) was documented. Donor cell (GFP+) chimerism and multilineage differentiation was assessed in blood (4, 12 and 24 weeks of recipient age), as well as in BM and spleen (24 weeks) (flow cytometry; FACS; Supplemental

Methods). Qualitative confirmation of engraftment results obtained by flow cytometry was achieved using immunohistochemistry (BM only; 6 months; Supplemental Methods).

In protocol 3, fetal and postnatal survival (up to 4 weeks) was documented. Donor cell (GFP+, CD45.1+) chimerism and multilineage differentiation was assessed in blood, BM and spleen at 4 weeks of recipient age by FACS. In addition, we looked into the hematopoietic progenitor cell/stem cell (Lin-) and HSC (LSK) containing compartments of donor cells found in BM (4 weeks; FACS; Supplemental Methods).

Statistical analyses

Results are expressed as mean±SEM, and statistical analysis was performed using 1- or 2-way ANOVA with Bonferroni post-hoc tests. A $p<0.05$ was considered significant. Statistical analysis was performed with Prism 6 statistical software (GraphPad Software Inc., La Jolla, CA).

Results

Nanoparticle characterization

Our protocol resulted in poly-disperse MLV-CHIR99021 solutions with nanoparticle diameter of 496±5nm, a loaded CHIR99021 mass of 27±1μg per 10^{10} particles, and the ability to release the inhibitor over 7 days; Supplemental Results; and Supplemental Figures 1 and 2). Using a nanoparticle-to-cell ratio of 600:1 for conjugation, we achieved a mean number of 55±8 MLV-CHIR99021 per BM-MNC or BM-HSC (Supplemental Results; Supplemental Figure 3). Based on this, we estimated that the mean CHIR99021 dose used was 0.15±0.02pg per cell. Preliminary studies demonstrated that conjugation of MLV to the cell surface of BM-MNC did not affect their ability to home to and engraft in fetal hematopoietic tissues post IUHCT (Figure 1, Supplemental Results and Supplemental Figure 4).

Conjugation of GSK3 inhibitor-loaded MLV on BM-MNC enhances long-term hematopoietic engraftment following in utero transplantation

Conjugation of MLV-CHIR99021 on BM-MNC and gradual release of the inhibitor (1.5μg per fetus) did not affect fetal survival, which contrasted with what we observed when the same amount of CHIR99021 was administered as a bolus at the time of IUHCT ($p<0.05$; Figure 2B). Donor cell engraftment in blood at 4 weeks of recipient age was similar between animals in group I and survivors in group II (group I: 15.5±1.4% vs. group II: 10.5±2.3%) but was more than three times higher in group III animals (group III: 52.9±2.8%; $p<0.0001$ vs. groups I and II) (Figure 2C, D). In contrast to groups I and II, engraftment in group III animals remained stable at 12 and 24 weeks (12 weeks: 48.3±2.2%; 24 weeks: 48.5±3.1%; $p<0.0001$ vs. groups I and II) (Figure 2D). There were no differences in white blood cell count between groups (group I: $4.5\pm0.4*10^3/\mu L$; group II: $5.3\pm0.5*10^3/\mu L$; group III: $4.9\pm0.3*10^3/\mu L$), multilineage analysis of donor and host cells did not demonstrate any differences in the relative levels of different lineages in the hematopoietic compartments of experimental offspring. (Figure 2E, F; Supplemental Figure 5 and 6A), and there was no evidence of graft-versus-host disease (GVHD) or malignancy. Similar results were observed in BM and spleen at 24 weeks (Figure 3; Supplemental Figures 6B and 7).

GSK3 inhibitor released by MLV increases engraftment following in utero transplantation by acting on hematopoietic stem/progenitor cells

BM-HSC were isolated and cultured over a 7-day period with (or without) a similar amount of CHI99021 to the one carried by MLV (per cell) added to the media. At the end of the culture period, approximately 25% of the harvested cells were LSK (Figure 4A), and this was not affected by the presence of CHIR99021. In agreement to what has been published previously, exposure of BM-HSC to CHIR99021 over the 7-day culture period resulted in significant increase in the total number of both BM-MNC and BM-HSC (Figure 4B), and was associated with modulation of the Wnt (downregulation of Axin2 expression, upregulation of Ccnd1 expression), Notch (upregulation of Hes1 expression), and Hedgehog (upregulation Gli3 and Ptch1 expression) pathways that have been shown to affect HSC function, proliferation kinetics and repopulating capacity (Figure 4C)[12-17]. Moreover, we found that BM-HSC cultured with

CHIR99021 had enhanced clonogenic potential evident in colony-forming unit (CFU) assays compared to untreated controls (Figure 4D, E). At the end of the culture and prior to IUHCT, BM-HSC from freshly isolated BM-MNC were replaced one-to-one with expanded BM-HSC (cultured with or without CHIR99021).

In agreement to our previous in vivo experiments, donor cell chimerism in blood following IUHCT of 10^7 BM-MNC was 14.3±1.9% at 4 weeks (group I; Figure 4H, I), and levels of engraftment gradually reduced during the follow-up period (Figure 4I). Replacement of BM-HSC from freshly isolated BM-MNC with expanded BM-HSC led to reduced blood engraftment at 4 weeks (group II: 4.9±0.6%, $p<0.05$ vs. group I) (Figure 4H, I), and only microchimerism thereafter (Figure 4I). Addition of CHIR99021 into the culture media of BM-HSC prevented the latter drop in chimerism at 4 weeks (group III: 12.9±1.6%, $p<0.05$ vs. group II) (Figure 4H, I), but did not enhance it any further (Figure 4I). Conjugation of MLV-CHIR99021 on donor BM-HSC (dose of CHIR99021: 1.5ng of per fetus) resulted in an impressive increase in baseline engraftment levels post IUHCT (4 weeks; Group V: 41.8±3.7%, $p<0.0001$ vs. Groups I to IV) (Figure 4H, I), and this remained unchanged up to 24 weeks of recipient age (Figure 4I). Conjugation of donor cells with empty particles (group IV) did not reproduce the chimerism rise seen in Group V animals (Figure 4H, I). More importantly, the levels of engraftment achieved by conjugating MLV-CHIR99021 only on BM-HSC were similar to those seen when all donor BM-MNC were "decorated" with MLV-CHIR99021. There were no differences in white blood cell count (group I: $4.7±0.2*10^3$/μL; group II: $4.3±0.6*10^3$/μL; group III: $5.2±0.4*10^3$/μL; group IV: $4.4±0.4*10^3$/μL; group V: $5.4±0.7*10^3$/μL) and multilineage analysis of donor and host cells between groups, (Figure 4J; Supplemental Figure 8), and there was no evidence of GVHD or malignancy. Similar results were seen in the BM and spleen at 24 weeks (Supplemental Figure 9).

Pseudo-autocrine bioactivity of GSK3 inhibitor released by MLV results in targeted augmentation of donor cell repopulating function

IUHCT of a 1:1 mixture of unmodified B6-GFP and B6-CD45.1 (CD45.1+) cells resulted in similar levels of engraftment in blood of recipients at 4 weeks of age [group I(GFP+): 14.7±2.2% vs. group I(CD45.1+): 12.1±1.7%; group I(GFP+/CD45.1+ ratio): 1.2±1.7] (Figure 5C, D).

Conjugation of MLV-CHIR99021 on either B6-GFP or B6-CD45.1 cells resulted in targeted enhancement of blood chimerism of the donor cell subpopulation carrying the inhibitor loaded particles [group II(GFP+): 33.2±2.6% vs. group II(CD45.1+): 10.2±1.4%, $p<0.001$; group II(GFP+/CD45.1+ ratio): 3.3±1.3, $p<0.0001$ vs. group I; group III(GFP+): 9.23±2.1% vs. group III(CD45.1+): 33.3±8.7%, $p<0.001$; group III(GFP+/CD45.1+ ratio): 0.3±1.1, $p<0.0001$ vs. groups I and II (Figure 5C, D). Multilineage analysis did not demonstrate any differences between groups (Figure 5E, F). Results in BM and spleen mirrored those in blood (Figure 5A; Supplemental Figures 10 and 11)

The proportion of donor Lin- cells (percentage of the sum of CD45.1+ and CD45.2+) was 2.8±0.2% in group I with no differences among experimental groups (Figure 6B). However, when we looked at the absolute number of donor Lin- cells (per hind limb long-bone) we saw a significant increase in hematopoietic progenitor number only in the donor cell subpopulation subjected to targeted GSK3 inhibition (Figure 6C, D). Similar effects were observed in the donor BM-MNC HSC-containing compartment (LSK cells). The proportion of donor "primitive" LSK cells (percentage of Lin- within the sum of CD45.1+ and CD45.2+ BM-MNC) was 11.2±0.6% in Group I, and no differences were observed among experimental groups (Figure 6E). However, when we estimated the absolute number of donor LSK cells we saw a significant rise in HSC number only in the donor cell subpopulation carrying MLV-CHIR99021 (Figure 6F, G).

Discussion

The data presented here demonstrate that temporally- and spatially-targeted GSK3 inhibition of BM-HSC results in remarkable levels of stable hematopoietic engraftment following experimental IUHCT. The gradual release of the inhibitor from liposome-based, synthetic nanoparticles conjugated on the surface of donor cells resulted in inhibition of GSK3 activity via a pseudo-autocrine mechanism. The targeted and sustained in vivo GSK3 inhibition, led to significant increases in overall cellularity of the donor cell pool post IUHCT, with an associated expansion of the donor hematopoietic progenitor and stem cell populations (Lin-/Sca1+/ckit+; LSK). The enhanced proliferation kinetics of cell-engineered BM-HSC mitigated the competitive advantage of fetal equivalents, with no evidence of increased differentiation and exhaustion of

BM-HSC with long-term repopulating capacity. Our study has significant translational potential as the levels of engraftment achieved following IUHCT of nanoparticle-conjugated donor cells in our murine model would be therapeutic for common congenital hematological disorders including thalassemia and sickle cell disease[32-35], allowing for "single-step" prenatal treatment[36].

The most compelling rationale for the utilization of IUHCT for the treatment of congenital diseases of hematopoiesis is based on unique opportunities related to normal developmental events that may facilitate engraftment and avoid complications associated with postnatal transplantation[1]. The most important of these is the development of the immune system, which allows the induction of "actively acquired" tolerance and hematopoietic engraftment of allogenic fetal (fetal liver)[37] and adult (BM-derived) cells[2] across immune barriers. Despite the potential advantages of IUHCT over postnatal transplantation, IUHCT in many animal models with intact fetal hematopoiesis has been limited by low levels of engraftment which are enough for tolerance induction, but inadequate for therapeutic effect for many target disorders[38-41].

The main cause of the latter is host cell competition. The first successful animal studies of IUHCT were performed in stem cell-defective murine models[42], and in immunodeficient mouse strains[43]. In the fetus with intact hematopoiesis during in utero development (as in inherited hemoglobinopathies), fetal stem cell and progenitor populations have a competitive advantage over their postnatal (adult) equivalents. As a result, IUHCT of large doses of donor cells (2 x 10^{11} adult BM-MNC/kg intravenously) results in rather modest levels of long-term chimerism of less than 10%[3;38]. Such engraftment would not be therapeutic for most congenital hematological disorders targeted by IUHCT, which would necessitate chimerism levels of around 20%[32-35;44]. Although current allogenic IUHCT protocols allow the induction of donor specific tolerance that could be combined with non-myeloablative postnatal HSC transplantation to achieve clinically relevant engraftment[7;45], the development of a "single-step" prenatal therapeutic strategy would be clinically, financially, and ethically desirable. In previous murine studies, we and others attempted to address this by enhancing donor BM-HSC homing to fetal hematopoietic niches[46], or by mobilization of host HSC from fetal hematopoietic niches thus generating more "space" for donor cells to engraft[47;48]. An additional approach that could be used in parallel with the latter strategies would be to attempt to make adult BM-HSC more "competitive" by enhancing the proliferation kinetics of donor cells. An appealing cellular target for such an approach would be GSK3.

In the seminal study by Trowbridge and colleagues, a 30mg/kg dose of the GSK3 inhibitor CHIR99021 was administered twice weekly to sub-lethally irradiated recipients of mouse or human BM-HSC over a period of 5 weeks, and resulted in improved proliferation kinetics of donor cells and enhanced hematopoietic repopulation[26]. The latter would not be feasible in IUHCT due to limited access to the fetus, and potential fetal toxicity of systemic GSK3 inhibition. An elegant solution to this problem was introduced by Stephan and colleagues, utilizing novel nanoparticle technology[27-29]. They described a strategy to enhance the efficacy of cell therapy via the conjugation of adjuvant drug–loaded nanoparticles to the cell surface. Multi-layered, liposome-based nanoparticles (multilamelar lipid vesicles; MLV) were formed with a hydrophilic core that allowed loading and slow-release of various bioactive chemicals, providing targeted and sustained pseudo-autocrine stimulation to donor cells. The results of the present experimental series extend these observations to the fetal hematopoietic environment and establish a strong salutary effect of sustained and targeted donor cell GSK3 inhibition in hematopoietic engraftment following IUHCT. Our experiments demonstrate that continuous but time-limited in vivo inhibition of GSK3 in donor cells, resulted in remarkable enhancement in engraftment levels following allogenic IUHCT. The near 50% chimerism achieved is reproducible, and more importantly stable for up to 6 months. The latter contrasts with control observations and would be consistent with GSK3 inhibition-mediated increase in the overall number of donor HSC with long-term repopulating capacity. Single, systemic administration of the same small amount of CHIR99021 to the fetus at the time of IUHCT resulted in increased fetal mortality and failed to reproduce the effects achieved by pseudo-autocrine release from MLV-CHIR99021. The reduction in fetal survival can be explained by well-recognized feto-toxic effects of "global" GSK3 inhibition[49], and the lack of benefit by the requirement for sustained GSK3 inhibition for therapeutically relevant effects to be manifest[21;26;50]. Our findings also confirmed previous observations[26] that the biological effect of GSK3 inhibition was specific to the primitive, HSC/HPC-containing subpopulation of LSK cells; cell-surface "decoration" of donor LSK cells with MLV-CHIR99021 resulted in similar levels of stable haematopoietic engraftment following IUHCT to those observed when nanoparticles are conjugated to the whole BM-MNC inoculum. This was despite the even smaller total dose of CHIR99021 administered and suggests that it is the total dose of CHIR99021 per cell (0.15pg per cell) that is of importance is this setting[27].

In our "competitive in utero repopulation" experiments, we adapted the protocols introduced by Harrison in 1980[31] and applied them in the setting of the competitively robust fetal hematopoietic environment. We demonstrated that targeted GSK3 inhibition, achieved by pseudo-autocrine activity of the inhibitor released from MLV-CHIR99021, offered a distinct competitive advantage to the donor cell population carrying the particles. This competitive boost was both against the "non-engineered" donor BM-MNC population, as well as fetal haematopoietic cells. In addition, we showed that the CHIR99021-induced increase in engraftment was associated with a proportional increase in the absolute number of donor HSC/HPC, consistent with augmented expansion of the HPC and HSC compartments of donor MNC (in number but not in proportion, due to improved cycling kinetics) as a possible explanation of the enhanced engraftment of cells "decorated" with MLV-CHIR99021. The stable levels of long-term hematopoietic engraftment observed in our 6-month follow-up studies, suggest that the sustained but transient inhibition of GSK3 expands both donor HSC and HPC pools without affecting the long-term repopulating capacity of HSC, although the latter would need to be confirmed by transplanting donor HSC to serial recipients. This contrasts with what has been found in assays of long-term HSC function, in which disruption of GSK3 progressively depleted HSC through activation of mammalian target of rapamycin (mTOR)[21;50]. Our findings also differ from the original observations by Trowbridge and colleagues, who showed that GSK3 inhibition does not induce expansion of the long-term repopulating HSC pool [26]. These differences can be attributed to the transient and brief GSK3 inhibition utilized in the present experimental series, as well as the very small dose of the inhibitor used. Potential human application may require larger CHIR99021 doses (per cell) and inhibitor release over a more prolonged period of time, which could result in mTOR activation-related effects on HSC. Such issues could be addressed by "decorating" the cell surface with nanoparticles containing both GSK3b inhibitors and rapamycin. Identification of the optimal dose/concentration of candidate growth factors/small molecule agents as well as the optimal duration of stimulation in human HSC (to achieve maximal in vivo donor HSC without affecting long-term repopulating capacity) would be essential prior to clinical translation.

Although we did not look into the direct molecular effects of GSK3 inhibition on BM-derived cells used in IUHCT experiments, 7-day culture of BM-derived LSK cells in the presence of a similar amount of CHIR99021 to that carried per cell in MLV-CHIR99021-conjugated LSK cells

resulted in increase in the number of primitive HSC (but not their proportion compared to lineage-committed hematopoietic cells). The observed enhanced proliferation kinetics were associated with modulation of the Wnt, Notch and Hedgehog pathways, similar to those observed by Trowbridge et al[26], as well as augmented ability compared to controls to form hematopoietic progenitor colonies in semi-solid media. It was not possible to utilize MLV-CHIR99021 in our culture experiments (serum free culture conditions do not allow gradual MLV degradation and inhibitor release). As a result, our in vitro observations provide only indirect evidence of a mechanism for the GSK3 inhibition-associated enhancement in HSC/HPC repopulating capacity via the modulation of cellular pathways known to regulate their homeostasis and proliferation kinetics[21;26;50]. More importantly, accurate determination of the exact molecular pathways that mediate the salutary effect of in vivo GSK3 inhibition in the setting of IUHCT would require transcriptomic analysis of isolated donor MLV-CHIR99021-carrying HSC soon after transplantation, as well as analysis of isolated long-term repopulating donor HSC (derived from the original "cell-engineered" donor HSC) at later timepoints. Such analyses were not performed in the present study but will be the focus of future work in our laboratory.

When 7-day-cultured, CHIR99021-pre-treated BM-HSC were used for IUHCT, they (as expected) out-performed control/untreated cultured equivalents, but failed to generate engraftment levels similar to those achieved when MLV-CHIR99021-"decorated" BM-HSC were used. Although these findings could be partly attributed to the well-recognized effects of culture to the long-term repopulating ability of HSC[51-53], they nonetheless support the requirement for sustained in vivo GSK3 inhibition of donor cells in order to achieve the remarkable improvement in engraftment. The MLV-CHIR99021 nano-delivery platform has unique properties that allow such targeted release of the inhibitor in the setting of IUHCT, and resulted in levels of long-term engraftment that would be therapeutic for many inherited diseases of hematopoiesis[32-35;44]. The scalability and adaptability (inhibitor loading mass and release profile) of the MLV-CHIR99021 platform, as well as the low associated costs (7 US dollars per 10^7 "engineered" cells) add further translational value to our findings.

We did not perform dedicated dose-determining, efficacy and toxicity studies in this experimental series, and the choice of CHIR99021 dose per cell was made based on previously published data[27]. Although the total dose of CHIR99021 administered per fetus varied from

6μg/kg to 6mg/kg, we saw no MLV-CHIR99021-associated toxic effects to the fetus at any inhibitor dose. Even though the maximum dose of the inhibitor used in our experiments was much lower than that used in previously published work in postnatal transplantation[26], a bolus 6mg/kg dose of CHIR99021 at the time of IUHCT resulted in increased fetal mortality. A similar CHIR99021 dose carried in MLV had no adverse effects on fetal survival. This as well as the lack of effect of the nanoparticles on homing of donor cells to fetal hematopoietic tissues, and on induction of central (thymic) immune tolerance highlight the favorable safety characteristics of the nano-delivery platform in the setting of IUHCT. Although we did not include secondary transplantation experiments in our experimental design and did not perform specific analysis for long-term repopulating HSC (CD150+, CD48+) within donor LSK cells, the minimal change in engraftment levels over the 24-week follow-up period observed when LSK cells "decorated" with MLV-CHIR99021 were used for IUHCT supports a salutary effect of the inhibitor on HSC with long-term repopulating capacity. However, serial transplantation experiments will be crucial to establish unequivocally the effect of our therapeutic platform on long-term repopulating HSC prior to clinical application to the human fetus. Finally, the observation of improved competitive capacity of MLV-CHIR99021 treated donor cells observed in this model of a non-myeloablated, highly competitive host hematopoietic system, has obvious implications beyond IUHCT for minimally myeloablative strategies in postnatal HSC transplantation.

Acknowledgments

We would like to thank the staff at the Metabolomic Core facility, Children's Hospital of Philadelphia for measuring CHIR99021 level in various experiments.

This work was supported by grants from the Wellcome Trust post-doctoral fellowship for MB/PhD graduates (ref. number: 098539/Z/12/Z) (S.P.L.); the National Institutes of Health Research (P.D.C.); and the CAPES agency, Brazil (ref. number: 99999.002599/2014-07) (C.G.F.).

Authorship Contributions

S.P.L., C.G.F., A.I.B.S.D., H.L., L.T., D.J.I., A.W.F. designed the work. S.P.L., C.G.F., A.I.B.S.D., H.L., A.G.K., J.D.V., J.D.S., N.J.A. performed the experiments and contributed to data collection. I.N. and I.N. developed the method and performed the measurements of CHIR99021. S.P.L., C.G.F., A.I.B.S.D., H.L., I.N., I.N., W.P., P.D.C., D.J.I., A.W.F. contributed to data analysis and interpretation. S.P.L., C.G.F., J.D.S., N.J.A prepared the figures. S.P.L., C.G.F., A.W.F. wrote the manuscript. S.P.L., C.G.F., A.I.B.S.D., H.L., J.D.S., N.J.A., I.N., A.F.M., J.L.M., W.P., P.D.C., D.J.I., A.W.F. contributed to the revision of the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

References

1. Loukogeorgakis SP, Flake AW. In utero stem cell and gene therapy: current status and future perspectives. Eur.J.Pediatr.Surg. 2014;24:237-245.

2. Kim HB, Shaaban AF, Milner R, Fichter C, Flake AW. In utero bone marrow transplantation induces donor-specific tolerance by a combination of clonal deletion and clonal anergy. J.Pediatr.Surg. 1999;34:726-729.

3. Boelig MM, Kim AG, Stratigis JD et al. The Intravenous Route of Injection Optimizes Engraftment and Survival in the Murine Model of In Utero Hematopoietic Cell Transplantation. Biol.Blood Marrow Transplant. 2016;22:991-999.

4. Vrecenak JD, Pearson EG, Santore MT et al. Stable long-term mixed chimerism achieved in a canine model of allogeneic in utero hematopoietic cell transplantation. Blood 2014;124:1987-1995.

5. Kim HB, Shaaban AF, Yang EY, Liechty KW, Flake AW. Microchimerism and tolerance after in utero bone marrow transplantation in mice. J.Surg.Res. 1998;77:1-5.

6. Peranteau WH, Heaton TE, Gu YC et al. Haploidentical in utero hematopoietic cell transplantation improves phenotype and can induce tolerance for postnatal same-donor transplants in the canine leukocyte adhesion deficiency model. Biol.Blood Marrow Transplant. 2009;15:293-305.

7. Peranteau WH, Hayashi S, Hsieh M, Shaaban AF, Flake AW. High-level allogeneic chimerism achieved by prenatal tolerance induction and postnatal nonmyeloablative bone marrow transplantation. Blood 2002;100:2225-2234.

8. Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. Biochem.J. 2001;359:1-16.

9. Yost C, Torres M, Miller JR et al. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev. 1996;10:1443-1454.

10. Jia J, Amanai K, Wang G et al. Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. Nature 2002;416:548-552.

11. Foltz DR, Santiago MC, Berechid BE, Nye JS. Glycogen synthase kinase-3beta modulates notch signaling and stability. Curr.Biol. 2002;12:1006-1011.

12. Murdoch B, Chadwick K, Martin M et al. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proc.Natl.Acad.Sci.U.S.A 2003;100:3422-3427.

13. Reya T, Duncan AW, Ailles L et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409-414.

14. Bhardwaj G, Murdoch B, Wu D et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat.Immunol. 2001;2:172-180.

15. Karanu FN, Murdoch B, Gallacher L et al. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J.Exp.Med. 2000;192:1365-1372.

16. Duncan AW, Rattis FM, DiMascio LN et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat.Immunol. 2005;6:314-322.

17. Ballin A, Lehman D, Sirota P, Litvinjuk U, Meytes D. Increased number of peripheral blood CD34+ cells in lithium-treated patients. Br.J.Haematol. 1998;100:219-221.

18. Boggs DR, Joyce RA. The hematopoietic effects of lithium. Semin.Hematol. 1983;20:129-138.

19. Joyce RA. Sequential effects of lithium on haematopoiesis. Br.J.Haematol. 1984;56:307-321.

20. Ricci P, Bandini G, Franchi P et al. Haematological effects of lithium carbonate: a study in 56 psychiatric patients. Haematologica 1981;66:627-633.

21. Huang J, Zhang Y, Bersenev A et al. Pivotal role for glycogen synthase kinase-3 in hematopoietic stem cell homeostasis in mice. J.Clin.Invest 2009;119:3519-3529.

22. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc.Natl.Acad.Sci.U.S.A 1996;93:8455-8459.

23. Hedgepeth CM, Conrad LJ, Zhang J et al. Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev.Biol. 1997;185:82-91.

24. Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr.Biol. 1996;6:1664-1668.

25. Phiel CJ, Klein PS. Molecular targets of lithium action. Annu.Rev.Pharmacol.Toxicol. 2001;41:789-813.

26. Trowbridge JJ, Xenocostas A, Moon RT, Bhatia M. Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. Nat.Med. 2006;12:89-98.

27. Stephan MT, Moon JJ, Um SH, Bershteyn A, Irvine DJ. Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. Nat.Med. 2010;16:1035-1041.

28. Stephan MT, Stephan SB, Bak P, Chen J, Irvine DJ. Synapse-directed delivery of immunomodulators using T-cell-conjugated nanoparticles. Biomaterials 2012;33:5776-5787.

29. Jones RB, Mueller S, Kumari S et al. Antigen recognition-triggered drug delivery mediated by nanocapsule-functionalized cytotoxic T-cells. Biomaterials 2017;117:44-53.

30. Merianos DJ, Tiblad E, Santore MT et al. Maternal alloantibodies induce a postnatal immune response that limits engraftment following in utero hematopoietic cell transplantation in mice. J.Clin.Invest 2009;119:2590-2600.

31. Harrison DE. Competitive repopulation: a new assay for long-term stem cell functional capacity. Blood 1980;55:77-81.

32. Hsieh MM, Fitzhugh CD, Tisdale JF. Allogeneic hematopoietic stem cell transplantation for sickle cell disease: the time is now. Blood 2011;118:1197-1207.

33. Andreani M, Testi M, Battarra M, Lucarelli G. Split chimerism between nucleated and red blood cells after bone marrow transplantation for haemoglobinopathies. Chimerism. 2011;2:21-22.

34. Walters MC, Patience M, Leisenring W et al. Stable mixed hematopoietic chimerism after bone marrow transplantation for sickle cell anemia. Biol.Blood Marrow Transplant. 2001;7:665-673.

35. Walters MC, Patience M, Leisenring W et al. Bone marrow transplantation for sickle cell disease. N.Engl.J.Med. 1996;335:369-376.

36. Peranteau WH, Hayashi S, Abdulmalik O et al. Correction of murine hemoglobinopathies by prenatal tolerance induction and postnatal nonmyeloablative allogeneic BM transplants. Blood 2015;126:1245-1254.

37. Nijagal A, Derderian C, Le T et al. Direct and indirect antigen presentation lead to deletion of donor-specific T cells after in utero hematopoietic cell transplantation in mice. Blood 2013;121:4595-4602.

38. Peranteau WH, Endo M, Adibe OO, Flake AW. Evidence for an immune barrier after in utero hematopoietic-cell transplantation. Blood 2007;109:1331-1333.

39. Nijagal A, Wegorzewska M, Jarvis E et al. Maternal T cells limit engraftment after in utero hematopoietic cell transplantation in mice. J.Clin.Invest 2011;121:582-592.

40. Lovell KL, Kraemer SA, Leipprandt JR et al. In utero hematopoietic stem cell transplantation: a caprine model for prenatal therapy in inherited metabolic diseases. Fetal Diagn.Ther. 2001;16:13-17.

41. Harrison MR, Slotnick RN, Crombleholme TM et al. In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. Lancet 1989;2:1425-1427.

42. Fleischman RA, Mintz B. Prevention of genetic anemias in mice by microinjection of normal hematopoietic stem cells into the fetal placenta. Proc.Natl.Acad.Sci.U.S.A 1979;76:5736-5740.

43. Blazar BR, Taylor PA, Vallera DA. In utero transfer of adult bone marrow cells into recipients with severe combined immunodeficiency disorder yields lymphoid progeny with T- and B-cell functional capabilities. Blood 1995;86:4353-4366.

44. Andreani M, Nesci S, Lucarelli G et al. Long-term survival of ex-thalassemic patients with persistent mixed chimerism after bone marrow transplantation. Bone Marrow Transplant. 2000;25:401-404.

45. Peranteau WH, Hayashi S, Kim HB, Shaaban AF, Flake AW. In utero hematopoietic cell transplantation: what are the important questions? Fetal Diagn.Ther. 2004;19:9-12.

46. Peranteau WH, Endo M, Adibe OO et al. CD26 inhibition enhances allogeneic donor-cell homing and engraftment after in utero hematopoietic-cell transplantation. Blood 2006;108:4268-4274.

47. Kim AG, Vrecenak JD, Boelig MM et al. Enhanced in utero allogeneic engraftment in mice after mobilizing fetal HSCs by alpha4beta1/7 inhibition. Blood 2016;128:2457-2461.

48. Witt RG, Wang B, Nguyen QH et al. Depletion of murine fetal hematopoietic stem cells with c-Kit receptor and CD47 blockade improves neonatal engraftment. Blood Adv. 2018;2:3602-3607.

49. Patorno E, Huybrechts KF, Hernandez-Diaz S. Lithium Use in Pregnancy and the Risk of Cardiac Malformations. N.Engl.J.Med. 2017;377:893-894.

50. Huang J, Nguyen-McCarty M, Hexner EO, Danet-Desnoyers G, Klein PS. Maintenance of hematopoietic stem cells through regulation of Wnt and mTOR pathways. Nat.Med. 2012;18:1778-1785.

51. Sauvageau G, Iscove NN, Humphries RK. In vitro and in vivo expansion of hematopoietic stem cells. Oncogene 2004;23:7223-7232.

52. Piacibello W, Sanavio F, Severino A et al. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 1999;93:3736-3749.

53. Zhang CC, Kaba M, Ge G et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat.Med. 2006;12:240-245.

Figure Legends

Figure 1: IUHCT of B6-GFP BM-MNC conjugated with empty MLV into Balb/c fetuses.

(A) Experimental design: effect of empty MLV conjugation on BM-MNC ability to home and engraft post IUHCT. Group I: unconjugated BM-MNC and group II: DiD labeled-empty MLV conjugated BM-MNC. (B) Fetal survival 4 days post IUHCT on both groups. Nanoparticle conjugation had no effect on fetal survival (Group I: 91% vs. Group II: 100%). (C) Qualitative assessment of donor cell homing to liver, spleen and thymus by fluorescent–stereotactic microscopy (4 days post IUHCT) demonstrated no differences between experimental groups. (D) Quantitative confirmation of donor cell engraftment (4 days post IUHCT) using flow cytometry [Group I(Liver): 20.6±2.1% vs. Group II(Liver): 18.3±1.9%; Group I(Spleen): 26.1±2.1% vs. Group II(Spleen): 27.7±2.9%; Group I(Thymus): 0.8±0.1% vs. Group II(Thymus): 0.7±0.1%].

Figure 2: **Conjugation of GSK3 inhibitor-loaded MLV on B6-GFP BM-MNC enhances long-term hematopoietic engraftment following IUHCT into Balb/c fetuses.**

(A) Experimental design: IUHCT of BM-MNC alone (group I), BM-MNC associated with a bolus of GSK3 inhibitor (group II) and BM-MNC conjugated with GSK3 inhibitor MLV (group III). (B) Fetal survival after 4 days post IUHCT on 3 groups - Group II: 38%; $p<0.05$ (‡) vs. Groups I (74%) and III (80%). There were no subsequent deaths. (C) Flow cytometry gating strategy for identifying CD45+ and GFP+ cells at P28 (light blue contour plots: CD45+, GFP- controls). (D) Donor cell levels in blood at P28, P84 and P168 after IUHCT into 3 groups. At 4

weeks, chimerism was similar between animals in Group I and survivors in Group II (Group I: 15.5±1.4% vs. Group II: 10.5%±2.3), but was more than three times higher in Group III animals that received donor BM-MNC carrying MLV-CHIR99021 (Group III: 52.9±2.8%; p<0.0001 (★) vs. Groups I and II) (Figure 2C, D). Engraftment levels in Group I and II offspring were significantly reduced at 12 and 24 weeks of age compared to 4 weeks [Group I(12 weeks): 5.9±0.7%, p<0.001 (†) vs. Group I(4 weeks); Group I(24 weeks): 3.4±0.7%, p<0.001(†) vs. Group I(4 weeks); Group II(12 weeks): 4.5±1.0%, p<0.001 (‡) vs. Group II(4 weeks); Group II(24 weeks): 2.9±0.9%, p<0.001(‡) vs. Group II(4 weeks)], but were maintained in Group III animals [Group III(12 weeks): 48.3±2.2%, p<0.0001(★) vs. Group I(12 weeks) and II(12 weeks); Group III(24 weeks): 48.5±3.1%, p<0.0001(★) vs. Group I(24 weeks) and II(24 weeks)] (E) Histograms for hematopoietic multilineage analysis using CD3, B220, Cd11b and Gr1 antibodies in blood at 6 months [light blue histograms: CD45+, GFP+ (for donor) or GFP- (for host), lineage specific antibody negative controls]. (F) Multilineage reconstitution of all 3 groups at 6 months, in blood, compared to donor lineages (GFP control = normal non-transplanted donor).

Figure 3: Long-term engraftment in bone marrow following IUHCT of B6-GFP BM-MNC conjugated with GSK3 inhibitor-loaded MLV into Balb/c fetuses.

(A) Flow cytometry gating strategy for identifying CD45+ and GFP+ cells in bone marrow in the 3 groups: BM-MNC alone (group I), BM-MNC associated with a bolus of GSK3 inhibitor (group II) and BM-MNC conjugated with GSK3 inhibitor MLV (group III) (light blue contour plots: CD45+, GFP- controls). (B) Percentage of CD45+ GFP+ donor cells on host bone marrow at 6 months in the 3 groups. Group I and II animals had similar levels of donor cell engraftment (Group I: 1.6±0.4% vs. Group II: 2.1±0.9%), while levels in Group III animals were almost twenty-five times higher (Group III: 50.4±2.6%, p<0.0001 (★) vs. Groups I and II). (C) Flow cytometry gating strategy for hematopoietic multilineage analysis using CD3, B220, Cd11b and Gr1 antibodies in bone marrow at 6 months (light blue histograms: lineage specific antibody negative controls). (D) Multilineage reconstitution of all 3 groups at 6 months, in bone marrow, compared to donor lineages, demonstrated no differences between groups. (E) Bone marrow

histology with immunohistochemistry for GFP (brown) at 6 months: in all 3 groups and Balb/c and GFP control mice.

Figure 4: GSK3 inhibitor released by multilamellar lipid vesicles increases engraftment following IUHCT by acting on hematopoietic stem/progenitor cells.

(A) BM-HSC were isolated and cultured over a 7-day period with (or without) a similar amount of CHI99021 to the one carried by MLV (per cell) added to the serum-free media. At the end of the culture period, approximately 25% of the harvested cells were LSK, and this was not affected by the presence of CHIR99021 in the media. Flow cytometry gating strategy for sorting B6-GFP BM-HSC (CD117+ and Sca1+ double positive cells shown; light blue contour plots: CD117- and Sca1- double negative controls). (B) Fold cell expansion after 7 days in culture on both groups (cultured without or with CHIR99021): total cells (left) and only in the HSC compartment (right). Exposure of BM-HSC to CHIR99021 over the 7-day culture period resulted in significant increase in the total number of both BM-MNC and BM-HSC ($p<0.05$) (★) (†). (C) Quantitative real time PCR on the HSC group cultured with CHIR 99021 demonstrated modulation of the Wnt (downregulation of Axin2 expression, upregulation of Ccnd1 expression), Notch (upregulation of Hes1 expression), and Hedgehog (upregulation Gli3 and Ptch1 expression) pathways that have been shown to affect HSC function, proliferation kinetics and repopulating capacity. (D) Hematopoietic CFU-assay: record of colonies growth. (E) Hematopoietic CFU (colony-forming unit) assays: comparing CFU numbers in the different lineages on both cultured groups (without or with CHIR99021). BM-HSC cultured in serum free media with CHIR99021 had enhanced clonogenic potential evident in CFU (semi-solid media) assays compared to untreated controls ($p<0.05$) (★). (F) Experimental design: group I – untreated BM-MNC, group II – BM-MNC with cultured HSC, group III – BM-MNC with HSC cultured with GSK3 inhibitor, group IV – BM-MNC in which fresh HSC were conjugated with empty MLV, group V – BM-MNC in which fresh HSC were conjugated with GSK3 inhibitor loaded MLV. Similar number of HSC contained on the isolated fresh bone marrow mononuclear cells (BM-MNC) was replaced by cultured HSC. The HSC population is usually 0,1% of all BM-MNC. Since, the fetus received 10^7 BM-MNC, this included 10,000 HSC, fresh, cultured or MLV decorated. (G) Fetal survival after 4 days post IUHCT into Balb/c fetuses in the 5 groups: there was no difference

between groups in fetal survival (Group I: 69% vs. Group II: 75% vs. Group III: 72%, vs. Group IV: 71%, Group V: 67%). (H) Density plots of a sample of each group demonstrating the gating strategy for the identification of the CD45+ GFP+ population (light blue contour plots: CD45+, GFP- controls). (I) Donor cell (B6-GFP) levels in blood at P28, P84 and P168 after IUHCT into 5 groups. Levels of engraftment gradually reduced during the follow-up period [Group I(12 weeks): 6.1±0.8%, $p<0.05$ (‡) vs. Group I(4 weeks): 14.3±1.9%; Group I(24 weeks): 3.9±1.1%, $p<0.05$ (‡) vs. Group I(4 weeks)]. Replacement of BM-HSC from freshly isolated BM-MNC with expanded BM-HSC resulted in reduced blood engraftment at 4 weeks [Group II(4 weeks): 4.9±0.6%, $p<0.05$ (†) vs. Group I(4 weeks)], and only microchimerism was detected thereafter. Addition of CHIR99021 into the culture media of BM-HSC prevented the latter drop in chimerism at 4 weeks [Group III(4 weeks): 12.9±1.6%, $p<0.05$ (†) vs. Group II(4 weeks)], but did not enhance it any further resulting in an engraftment profile in blood similar to that observed in animals that received IUHCT of unprocessed/freshly-isolated BM-MNC (group I). Conjugation of MLV-CHIR99021 on donor BM-HSC resulted in an impressive increase in baseline engraftment levels post IUHCT [Group V(4 weeks): 41.8±3.7%, $p<0.0001$ (★) vs. Groups (4 weeks) I to IV], and this remained unchanged up to 24 weeks of recipient age [Group V(12 weeks): 41.4±2.6%, $p<0.0001$ (★) vs. Group I(12 weeks) to IV(12 weeks); Group V(24 weeks): 45.4±2.3%, $p<0.0001$(★) vs. Group I(24 weeks) to IV(24 weeks)]. The effect of MLV-CHIR99021 on engraftment was due to the gradual release and activity of the inhibitor, as conjugation of donor cells with empty particles did not reproduce the chimerism rise seen in Group V animals [Group IV(4 weeks):19.2±2.9%, $p<0.0001$(★) vs. Group V(4 weeks), $p<0.05$ (†) vs. Group II(4 weeks), $p<0.05$ (‡) vs. Group IV(12 weeks) and Group IV(24 weeks)]. (J) Multilineage reconstitution of all 5 groups at 6 months, in blood, compared to donor lineages, did not demonstrate any differences between groups.

Figure 5: Pseudo-autocrine bioactivity of GSK3 inhibitor released by MLV results in targeted augmentation of donor cell repopulating function.

(A) Experimental design: IUHCT into Balb/c fetuses of a 1:1 mixture of B6GFP and B6-CD45.1 BM-MNC with both populations unconjugated (group I), with only B6GFP cells conjugated to GSK3 inhibitor loaded MLV (group II) and with only B6-CD45.1 cells conjugated (group III).

(B) Fetal survival after 4 days post IUHCT on 3 groups: there was no difference between groups (Group I: 71% vs. Group II: 68% vs. Group III: 69%). (C) Flow cytometry gating strategy for determining the percentage of both populations in blood: CD45.1+ (B6-CD45.1 cells) and GFP+ (B6-GFP cells) within the CD45 population, samples from the 3 groups (Light blue contour plots: 45.1- and 45.2- double negative controls; light blue histograms: GFP- or CD45.1- negative controls). (D) Mean percentage of GFP+ and CD45.1+ donor cells within groups I, II and III on blood. Group I showed similar levels of engraftment in blood of recipients at 4 weeks of age [Group I(GFP+): 14.7±2.2% vs. Group I(CD45.1+): 12.1±1.7%; Group I(GFP+/CD45.1+ ratio): 1.2±1.7]. Conjugation of MLV-CHIR99021 on either B6-GFP or B6-CD45.1 cells resulted in targeted enhancement of blood chimerism of the donor cell subpopulation carrying the inhibitor loaded particles. On group II, the percentage of GFP+ cells were significantly higher than CD45.1+ cells [Group II(GFP+): 33.2±2.6% vs. Group II(CD45.1+): 10.2±1.4%, $p<0.001$ (**§‡**); Group II(GFP+/CD45.1+ ratio): 3.3±1.3, $p<0.0001$ (★) vs. Group I(GFP+/CD45.1+ ratio)]. And on group III, the opposite was found [Group III(GFP+): 9.23±2.1% vs. Group III(CD45.1+): 33.3±8.7%, $p<0.001$ (■●); Group III(GFP+/CD45.1+ ratio): 0.3±1.1, $p<0.0001$ (†) vs. Group I(GFP+/CD45.1+ ratio) and Group II(GFP+/CD45.1+ ratio)]. (E) (F) Multilineage analysis in GFP+ (E) and CD45.1+ (F) donor cells, no differences between groups in the proportion of lymphoid (CD3+, B220+) and myeloid (CD11b+, Gr-1+) cells in blood.

Figure 6: Effect of targeted pseudo-autocrine bioactivity of GSK3 inhibitor released by MLV on donor hematopoietic stem/progenitor cells.

(A) Flow cytometry gating strategy for determining the hematopoietic progenitor cell/stem cell (Lin-) and HSC-containing compartments of donor cells found in BM of IUHCT recipients (Balb/c fetuses), samples from the 3 groups (Light blue contour plots: 45.1- and 45.2- double negative controls or CD117- and Sca1- double negative controls; light blue histograms: Lin-, GFP- or CD45.1- negative controls). (B) The proportion of donor Lin- cells (expressed as a percentage of the sum of CD45.1+ and CD45.2+ BM-MNC) was 2.8±0.2% in Group 1, and no differences were observed among experimental groups [Group II(Lin-): 3.1±0.3% vs. Group III(Lin-): 3.3±0.4%] (C) Absolute number of donor Lin- cells (per hind limb long-bone), significant increase in hematopoietic progenitor number only in the donor cell subpopulation

subjected to targeted GSK3 inhibition. [Group I(Lin-, GFP+): $2.5*10^3 \pm 0.4*10^3$ GFP+ cells vs. Group I(Lin-, CD45.1+): $4.8x10^3 \pm 0.5x10^3$ CD45.1+ cells; Group II(Lin-, GFP+): $16.7x10^3 \pm 1.9x10^3$ GFP+ cells vs. Group II(Lin-, CD45.1+): $5.7x10^3 \pm 1.5x10^3$ CD45.1+ cells, $p<0.05$ (§‡); Group III(Lin-, GFP+): $2.5x10^3 \pm 0.3x10^3$ GFP+ cells vs. Group III(Lin-, CD45.1+): $21.2x10^3 \pm 5.2x10^3$ CD45.1 cells, $p<0.001$ (■●)] . (D) Ratio of GFP+/CD45.1+ Lin- cells: dominance of the subpopulation that was MLV conjugated (⋆) (†). (E) Proportion of donor "primitive" LSK cells (expressed as a percentage of the Lin- cells in the sum of CD45.1+ and CD45.2+ BM-MNC), no differences were observed among experimental groups [Group 1(LSK) 11.2±0.6% vs. Group II(LSK): 9.8±1.5% vs. Group III(LSK): 8.8±1.4%]. (F) Absolute number of LSK cells (per hind limb long-bone), significant increase only in the donor cell subpopulation carrying MLV-CHIR99021 [Group I(LSK): $3.1x10^2 \pm 0.6x10^2$ GFP+ cells vs. Group I(LSK): $4.9x10^2 \pm 0.7x10^2$ CD45.1+ cells; Group II(LSK): $16.1x10^2 \pm 1.6x10^2$ GFP+ cells vs. Group II(LSK): $6.1*10^2 \pm 1.9*10^2$ CD45.1cells, $p<0.05$ (§‡); Group III(LSK): $2.4x10^2 \pm 0.7x10^2$ GFP+ cells vs. Group III(LSK): $18.8x10^2 \pm 6.2x10^2$ CD45.1 cells, $p<0.001$ (■●)]. (G) Ratio of GFP+/CD45.1+ LSK cells: showing the same dominance of the subpopulation that was MLV conjugated (★) (†⊕).