Deficiency of GRP94 in the Hematopoietic System Alters Proliferation Regulators in Hematopoietic Stem Cells

Biquan Luo,¹ Chun-Chih Tseng,¹ Gregor B. Adams,² and Amy S. Lee¹

We have previously reported that acute inducible knockout of the endoplasmic reticulum chaperone GRP94 led to an expansion of the hematopoietic stem and progenitor cell pool. Here, we investigated the effectors and mechanisms for this phenomenon. We observed an increase in AKT activation in freshly isolated GRP94-null HSC-enriched Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) cells, corresponding with higher production of PI(3,4,5)P₃, indicative of PI3K activation. Treatment of GRP94-null LSK cells with the AKT inhibitor MK2206 compromised cell expansion, suggesting a causal relationship between elevated AKT activation and increased proliferation in GRP94-null HSCs. Microarray analysis demonstrated a 97% reduction in the expression of the hematopoietic cell cycle regulator *Ms4a3* in the GRP94-null LSK cells, and real-time quantitative PCR confirmed this down-regulation in the LSK cells but not in the total bone marrow (BM). A further examination comparing freshly isolated BM LSK cells with spleen LSK cells, as well as BM LSK cells cultured in vitro, revealed specific down-regulation of *Ms4a3* in freshly isolated BM GRP94-null LSK cells. On examining cell surface proteins that are known to regulate stem cell proliferation, we observed a reduced expression of cell surface connexin 32 (Cx32) plaques in GRP94-null LSK cells. However, suppression of Cx32 hemichannel activity in wild-type LSK cells through mimetic peptides did not lead to increased LSK cell proliferation in vitro. Two other important cell surface proteins that mediate HSC-niche interactions, specifically Tie2 and CXCR4, were not impaired by *Grp94* deletion. Collectively, our study uncovers novel and unique roles of GRP94 in regulating HSC proliferation.

Introduction

THE SELF-RENEWAL OF HEMATOPOIETIC stem cells (HSCs) is tightly regulated by intrinsic determinants and extrinsic cues from the microenvironment [1]. Intrinsic determinants of HSC self-renewal and differentiation include cell cycle regulators, transcription factors, and chromatin-associated factors [2]. One specific intrinsic regulator of HSC self-renewal and differentiation is AKT, a serine/threonine kinase. Activated growth factor receptors recruit PI3K to the plasma membrane, allowing for the phosphorylation of phosphoinositides and conversion of PI(4,5)P₂ to PI(3,4,5)P₃. AKT, through binding to the PI(3,4,5)P₃ lipid products, localizes to the cell membrane and becomes activated. AKT is a major effector of the PI3K pathway, and many of its substrates regulate cell survival and growth [3]. The deletion of PTEN, which is a negative regulator of PI3K-AKT pathway in the mouse hematopoietic system, results in HSC hyperproliferation, myeloproliferative disorder, and leukemia [4,5]. Constitutive activation of AKT signaling causes short-term expansion of the hematopoietic stem and progenitor compartment through increased cycling and eventually leads to HSC depletion and leukemia [6].

While well-established cell cycle regulators such as p53 and p21^{cip1/waf1} are known to modulate HSC cell fate [7], novel hematopoietic cell cycle modulators have also been identified, including MS4A3 (HTm4) [8]. MS4A3 is a transmembrane protein of the MS4A family expressed in hematopoietic cells and other select cell types and tumors [9]. MS4A3 interacts with the cyclin-dependent kinase 2 (CDK2), cyclin A, and CDK-associated phosphatase complex, and its overexpression in hematopoietic cells has been reported to cause cell cycle arrest at the G₀/G₁ phase [10]. Thus, MS4A3 can potentially regulate HSC proliferation in vivo.

Extrinsic signals from the microenvironment control the expression of intrinsic determinants of HSC self-renewal and differentiation. HSCs reside in a specialized microenvironment known as the HSC niche which composes cellular and humoral signaling cues that regulate the survival, self-renewal, migration, differentiation, and quiescence of HSCs [11–13]. The first identified HSC niche was the bone marrow (BM) endosteal niche in which a specific type of osteoblastic cell represents the major component. More recently, endothelial cells and mesenchymal stem cells have also been identified to comprise a HSC niche and to regulate stem cell

¹Department of Biochemistry and Molecular Biology, USC Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California.

²Department of Cell and Neurobiology, Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, California.

physiology [14–17]. Local extrinsic elements from the niche include soluble factors that function through interactions with their receptors, such as SDF-1/CXCR4 [18], angiopoietin/Tie2 [19], Ca^{2+} /CaR [20], as well as direct contact through extracellular matrix and cell surface proteins [21,22], such as integrins [23,24]. Gap junction proteins have been shown to play important roles in HSC homeostasis. Connexin 43 (Cx43) in the endosteal niche is a crucial regulator of HSC homing and migration in an irradiated microenvironment [25], while connexin 32 (Cx32) is also required for maintaining hematopoietic progenitors in the BM. Indeed, it has been reported that Cx32^{-/-} mice showed expansion of BM Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) cells and increased LSK cell proliferation [26].

We previously identified an endoplasmic reticulum (ER) chaperone glucose-regulated protein (GRP94) as a novel regulator of HSCs and their interaction with the adult BM endosteal niche. As a chaperone that assists in the folding, assembly, and secretion of a selective collection of client proteins, GRP94 performs unique functions in the ER, and controls specific pathways critical for cell growth, differentiation, organ homeostasis, and immune functions [27–29]. Our previous study using an *Mx-1-Cre*-mediated inducible knockout mouse model identified GRP94 as a regulator for HSC physiology, as loss of GRP94 in the hematopoietic system leads to an expansion of the hematopoietic stem and progenitor cell pool through increased proliferation [30]. This increased proliferation could, in part, be attributed to detachment of HSCs from the BM niche, as GRP94-null HSCs show increased mobilization, as well as impaired homing and engraftment [30]. Furthermore, this impaired homing and engraftment was cell autonomous, as transplanting wild-type hematopoietic cells into a GRP94-null microenvironment yielded a normal hematopoietic profile with comparable numbers of HSCs as compared with controls. Thus, these earlier results indicated that loss of GRP94 function in the hematopoietic stem and progenitor cells activate cell intrinsic mechanisms which lead to the loss of quiescence and cell expansion in the GRP94-null mouse HSCs. Here, we explored these mechanisms in LSK cells isolated from the inducible *Grp94* knockout (*Grp94*^{fl/f}; *Mx-1-Cre*) mice. We discovered that GRP94 deficiency in LSK cells resulted in increased PI(3,4,5)P3 formation and AKT activation, and suppressing AKT activation with an allosteric AKT inhibitor compromised the increase of the GRP94-null LSK cells in vitro. A microarray analysis further revealed that GRP94 deficiency in freshly isolated LSK cells leads to a 97% reduction in *Ms4a3* mRNA expression. Further experimentation showed that the expression of *Ms4a3* is dependent on cell type and microenvironment. In addition, we observed decreased cell surface expression of Cx32 plaques in GRP94-null LSK cells. However, suppressing the hemichannel activity of Cx32 did not lead to increased LSK cell proliferation in vitro. Collectively, these identified pathways expand our knowledge on pleiotropic functions of GRP94 in regulating HSC proliferation.

Materials and Methods

Mice

Grp94^{fl/f}; *Mx-1-Cre* mice in a mixed C57BL/6; 129/Sv background were generated, and genotyping was performed

as previously described [30]. Littermates that were negative for *Cre* transgene were used as controls. Mice that were 5.5 to 6.5 weeks old were subjected to pI.pC-induced deletion of *Grp94* primarily in the hematopoietic system performed as previously described [30].

Reagents

The AKT inhibitor MK2206 [31] was purchased from Tocris Bioscience. MK2206 was dissolved in DMSO and diluted with DMEM before use. The Cx32 mimetic peptide and the control peptide with scrambled sequence were purchased from GenScript. The sequences of the peptides are as follows: for ³²Gap27, SRPTEKTVFT (extracellular loop 2, position 182–191 of Cx32 [32]); for scrambled control, TFEPIRISITK [33].

Flow cytometry

Flow cytometry was performed as previously described [30]. To identify hematopoietic stem and progenitor cells, Lineage [Lin; which consists of B220 (RA3-6B2), TER119 (TER119), CD4 (RM4-5), CD8a (53–6.7), Gr-1 (RB6-8C5), Mac-1 (WT.5) and CD41 (eBioMWR30 from eBioscience)], c-Kit (2B8), Sca-1 (D7; all from BD Pharmingen except CD41), CD150 (mShad150), and CD48 (HM48-1; both from eBioscience) were used. Antibodies against Tie2 (TEK4; eBioscience) and CXCR4 (12G5; BD Pharmingen) were used individually to detect cell surface Tie2 and CXCR4. Cell population analysis was performed on a BD FACS LSR II.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting was performed as previously described [30]. Primitive hematopoietic stem and progenitor cells as well as cells of different lineages in the BM were purified using an FACS Aria flow cytometer (Becton Dickinson) based on established cell surface phenotypes.

Cell culture

For culture of LSK cells, human stromal cells (HS-5) were irradiated at 15 Gy to stop proliferation before being seeded into 24-well plates and cultured overnight. Equal number of sorted LSK cells were plated in wells with or without HS-5 cells and cultured in α -MEM with 10% serum and 1% penicillin/streptomycin (P/S) for 48 h in the presence of IL-3 (10 ng/mL), IL-7 (50 ng/mL), SCF (100 ng/mL), and TPO (100 ng/mL). LSK cells cultured without HS-5 cells were harvested and enumerated with a hemacytometer. LSK cells co-cultured with HS-5 cells were harvested with medium, whereas HS-5 cells were trypsinized, collected, and mixed with the LSK cells. The mixture of cells was stained with hematopoietic cell marker CD45 (30-F11; eBioscience), and the number of CD45⁺ cells was calculated based on the proportion of CD45⁺ cells in the mixture and the number of HS-5 cells seeded.

U266 human multiple myeloma cells and HL60 human promyelocytic leukemia cells were maintained in RPMI 1640 with 10% FBS and 1% P/S, transduced with lentiviral shRNA against scrambled control sequence (shCtrl) or *Grp94* (shGrp94; Open Biosystems), and selected with puromycin (6 μ g/mL) for 1 week post transduction before being harvested for analysis.

Real-time quantitative reverse transcription–polymerase chain reaction

Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described [30]. The following primers were used: mouse *Ms4a3*, 5′-TAC TGC AAG CCC TCG GGG CCA-3′ and 5′-GGG TTT CTC CCT GCG GCA ACA-3′; human *Ms4a3*, 5′-AAG CCT GAA GCC TCC AAG TTC-3′; mouse and human *18S RNA*, 5′-CCA CTC CCG ACC CGG GGA GGT AGT GAC GAA-3′, and 5′-CTC AGC TAA GAG CAT CGA GGG GGC GCC GAG AGG-3′.

Immunofluorescence and confocal microscopy

Sorted BM LSK cells were washed and resuspended in serum-free DMEM (high glucose). Droplets of cells were then seeded on Superfrost Plus® Gold slides (FT-4981-GLPLUS; Thermo Scientific) and cultured at 37°C for 1 h. The cells were then fixed in 4% paraformaldehyde at room temperature (RT) for 10 min, then dehydrated by serial ethanol solutions, and allowed to dry. For immunostaining, the cells were rehydrated in PBS and then quenched with 0.25% (w/v) ammonium chloride in PBS containing 0.01% saponin (PBS/saponin) at RT for 5 min. The cells were then briefly permeabilized by 0.1% Triton X-100 in PBS/saponin and quenched again with 0.25% (w/v) ammonium chloride. After briefly washing with PBS/saponin, the cells were pre-blocked with 1% BSA in PBS/saponin (PBS/BSA/saponin) at RT for 1 h. For PI(3,4,5)P3 staining, endogenous mouse IgG was blocked with additional treatment with M.O.M.TM Mouse Ig Blocking Reagent (BMK-2202; Vector Laboratories) at RT for 1 h. Then, the cells were incubated with primary antibodies at 37°C for 1 h followed by secondary antibodies at 37°C for 30 min in 5% PBS/BSA/saponin. For PI(3,4,5)P3 staining, mouse anti-PI(3,4,5)P3 (20 µg/mL, Z-P345b; Echelon Biosciences) primary antibody and Alexa-fluor 488-conjugated goat anti-mouse secondary antibody (20 µg/mL; Life Technologies) were used. For phosphorylated-AKT (pAKT) staining, rabbit anti-pAKT (4 µg/mL, Ser473; Cell Signaling Technology) primary antibody and Alexa-fluor 488-conjugated goat anti-rabbit secondary antibody (20 µg/mL, Life Technologies) were used. For total AKT staining, mouse anti-pan-AKT (4 µg/mL, Cell Signaling Technology) primary antibody and Alexa-fluor 594-conjugated goat anti-mouse secondary antibody (20 µg/mL, Life Technologies) were used. For Cx32 staining, rat anti-Cx32 antiserum (1:1; Developmental Studies Hybridoma Bank) primary antibody and Alexa-fluor 488-conjugated goat anti-rat secondary antibody (20 µg/mL; Life Technologies) were used. The cells were then washed with PBS/saponin and ddH₂O and mounted with Vectashield antifade medium with DAPI (H-1200; Vector Laboratories).

Confocal images were obtained by a Zeiss LSM510 confocal microscope equipped with a Hamamatsu R6357 photomultiplier and LSM 510 Version 4.2 SP1 acquisition software (Carl Zeiss). Images of pAKT and total AKT were taken with a Plan-Apochromat 100×/1.4 oil DIC objective at 2× zoom. Images for pAKT and total AKT quantification were taken under 40×/1.30 oil objective without zoom, and quantified using ImageJ. Compressed Z-stack PI(3,4,5)P3 images were taken with an EC Plan-Neofluar

40×/1.30 oil objective without zoom. The same number of sections were compressed for each condition. Cx32 images were taken with a Plan-Apochromat 100×/1.4 oil DIC objective at 8× zoom.

Scrape-loading dye transfer assay

Gap junction permeability was determined using a Lucifer yellow scrape-loading technique [34]. HeLa cells that are deficient of all connexin expression [35] were transfected with Cx32 overexpression plasmid or the pcDNA3 empty vector by Bio-T transfection reagent (Bioland Scientific) for 24 h. The Cx32 expression plasmid was constructed by subcloning the mouse Cx32 cDNA amplified via RT-PCR from mouse liver RNA into the pcDNA3 expression vector. Confluent cells were incubated with scrambled peptide or Cx32 mimetic peptide ³²Gap27 for 30 min, washed with TBS, and loaded with mixture of Lucifer yellow and Rhodamine dextran followed by scratches using 20 µL peptide tips. After incubation at 37°C for 10 min, the cells were washed seven times with TBS before examination by a fluorescent microscope. Images were obtained by a Nikon ECLIPSE TE300 Inverted Microscope equipped with Hamamatsu Orca photomultiplier and MetaMorph acquisition software.

Statistics

Statistical significance was assayed by Student's *t*-test.

Results

GRP94 deficiency expands long-term HSC pool in the BM

Using the *Grp94^{fl/fl}; Mx-1-Cre* (*cGrp94^{fl/fl}*) mouse model that we previously created in which GRP94 is depleted in the hematopoietic system on administration of pLpC to 5.5 to 6.5 week-old mice [30], we determined the effect of GRP94 depletion on the primitive hematopoietic cell pool using the SLAM markers (CD150, CD48, and CD41). Littermates lacking the *Cre* transgene (*Grp94^{fl/fl}*) that are phenotypically equivalent to animals with wild-type *Grp94* alleles were also injected with pLpC and served as controls. There was a two-fold expansion of the long-term HSC (LT-HSC) enriched Lin[−] c-Kit⁺ Sca-1⁺ CD41[−] CD48[−] CD150⁺ (LSKCD41[−] CD48[−] CD150⁺) cell population (Fig. 1A, B), correlating with a two-fold increased hematopoietic stem and progenitor cells enriched Lin[−] Sca-1⁺ c-Kit⁺ (LSK) population (Fig. 1C). Interestingly, using the SLAM markers, the short-term HSC (ST-HSC)-enriched Lin[−] Sca-1⁺ c-Kit⁺ CD41[−] CD48[−] CD150[−] (LSKCD41[−] CD48[−] CD150[−]) cell population was not significantly altered (Fig. 1B).

We had previously observed the loss of quiescence and increased proliferation in *cGrp94^{fl/fl}* BM LSK cells determined by cell cycle analysis [30]. To study this further, we examined the expansion of the cells in vitro after stimulation with a defined cocktail of cytokines. When an equal number of *cGrp94^{fl/fl}* and *Grp94^{fl/fl}* BM LSK cells were seeded and cultured in medium containing IL-3, IL-7, SCF, and TPO for 2 days (Fig. 2A, top panel), the number of *cGrp94^{fl/fl}* hematopoietic cells generated showed a two-fold increase over the *Grp94^{fl/fl}* control (Fig. 2B). Under co-culture conditions with the HS-5 human stromal cell line for 2 days (Fig. 2A, lower panel), a

FIG. 1. Expanded primitive hematopoietic cell pools on GRP94 deficiency in the BM. **(A)** Representative flow cytometric analysis of BM cells using Lin, c-Kit, Sca-1, CD41, CD48, and CD150. **(B)** Quantification of flow cytometric analysis of LSKCD41⁻CD48⁻CD150⁺ LT-HSCs and LSKCD41⁻CD48⁻CD150⁻ ST-HSCs ($n = 12$ for *Grp94^{fl/fl}*, $n = 8$ for *cGrp94^{fl/fl}*). **(C)** Quantification of flow cytometric analysis of LSK cells in BM ($n = 12$ for *Grp94^{fl/fl}*, $n = 8$ for *cGrp94^{fl/fl}*). All data are presented as mean $\pm$ S.E., *** $P < 0.001$. BM, bone marrow; HSCs, hematopoietic stem cells; LSK, Lin⁻ Sca-1⁺ c-Kit⁺; LT-HSCs, long-term HSCs; ST-HSCs, short-term HSCs. Color images available online at www.liebertpub.com/scd

similar increase in *cGrp94^{fl/fl}* CD45⁺ hematopoietic cells was also observed following equal seeding of LSK cells from the two genotypes (Fig. 2C, D). These results confirm that loss of GRP94 in primitive hematopoietic cells leads to increased cell expansion under defined cell culture conditions.

Increased PI(3,4,5)P3 production and AKT activation in primitive hematopoietic cells on *Grp94* deletion

AKT activation is a major contributing factor for HSC proliferation. To investigate the underlying mechanisms for cell expansion due to GRP94 deficiency, we examined the activation of AKT in the primitive hematopoietic compartment using freshly isolated BM LSK cells from the two genotypes. Representative immunofluorescence staining images for pAKT and total AKT in the LSK cells are shown in Fig. 3A, C, and the level of staining was quantified and compared between the two genotypes (Fig. 3B, D). Our results revealed that while *Grp94^{fl/fl}* control LSK cells exhibited low levels of pAKT staining, *cGrp94^{fl/fl}* LSK cells showed significantly higher (1.8-fold) AKT phosphorylation. Interestingly, total AKT level was about 15% lower in the GRP94-null LSK cells compared with *Grp94^{fl/fl}* controls.

To determine at which step GRP94 regulates AKT activation in the LSK cells, we measured the effect of GRP94 knockout on the production of PI(3,4,5)P3, which is an indication of PI3K activity upstream of AKT activation.

PI(3,4,5)P3 production was measured by confocal microscopy after staining of purified LSK cells with a monoclonal anti-PI(3,4,5)P3 antibody previously established for sensitive detection of PI(3,4,5)P3 levels in cells, including hematopoietic cells [36]. Representative immunofluorescent images of LSK cells from the two genotypes are shown in Fig. 3E. While the staining pattern and intensity showed some heterogeneity among the LSK cells, *cGrp94^{fl/fl}* LSK cells exhibited a higher level of PI(3,4,5)P3 production, corresponding with the pAKT staining pattern. On quantitation, we observed approximately 80% of *cGrp94^{fl/fl}* LSK cells as positive for PI(3,4,5)P3 production compared with approximately 45% for the control *Grp94^{fl/fl}* LSK cells. Thus, GRP94 deficiency activates AKT in LSK cells in part via higher PI(3,4,5)P3 production.

To determine the requirement of AKT activity on *cGrp94^{fl/fl}* LSK cell proliferation, we isolated the *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* LSK cells and cultured them for 2 days in the presence or absence of an allosteric AKT inhibitor MK2206 [31]. We recently reported that MK2206 was able to block AKT phosphorylation in various cell lines cultured in vitro at concentrations as low as 50 nM [37]. As expected, *cGrp94^{fl/fl}* LSK cells nearly tripled in cell number during culture when treated with DMSO alone. MK2206 treatment compromised the cell expansion in a dose-dependent manner, with an effect readily observable at 50 nM of drug concentration (Fig. 3F). Thus, AKT activation contributes at least in part to *cGrp94^{fl/fl}* LSK cell hyperproliferation.

A w/o stromal cells: **w/ stromal cells:**

FIG. 2. In vitro culture of GRP94-null LSK cells. **(A)** Scheme of culturing Grp94^{f/f} and cGrp94^{f/f} LSK cells in vitro. (Upper panel) with growth factors and cytokines only; (lower panel) with stromal cells, growth factors, and cytokines. CD45 staining was used to determine hematopoietic cells derived from the LSK cells. **(B)** Cell numbers from cultures without stromal cells determined by a hemocytometer. **(C)** Flow cytometric analysis using CD45 as a marker for hematopoietic cells derived from LSK cells co-cultured with stromal cells. The green line represents Grp94^{f/f} cells, and the red line indicates cGrp94^{f/f} cells. **(D)** Quantification of hematopoietic cells under co-culture conditions with stromal cells, based on the number of stromal cells seeded and the proportion of CD45⁺ cells determined by flow cytometric analysis. All data are presented as mean $\pm$ S.E., ** $P < 0.01$. Color images available online at www.liebertpub.com/scd

Cell cycle regulator *Ms4a3* is down-regulated in GRP94-depleted LSK cells

To explore other mechanisms that may contribute to HSC expansion on Grp94 deletion, we isolated mRNA from cGrp94^{f/f} and Grp94^{f/f} LSK cells and performed a microarray analysis (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). The mRNA levels of major cell cycle regulators of HSCs such as p18, p19, p21, p27, and p57 in cGrp94^{f/f} LSK cells were comparable with Grp94^{f/f} controls. However, the expression of a newly identified cell cycle regulator *Ms4a3* [8] was reduced to approximately 3% of the control (Supplementary Table S2). The reduced expression of *Ms4a3* in cGrp94^{f/f} LSK cells was confirmed by real-time quantitative PCR, as the expression of *Ms4a3* in cGrp94^{f/f} LSK cells was 10% of that in Grp94^{f/f} LSK cells (Fig. 4A). Interestingly, the reduced expression of *Ms4a3* mRNA level on Grp94 deletion was only observed in LSK cells and not in the whole BM or cells of specific lineages (including Gr-1⁺, F4/80⁺ and B220⁺ cells) from cGrp94^{f/f} mice (Fig. 4A), suggesting that the regulatory effects of GRP94 on *Ms4a3* may be restricted to the primitive hematopoietic cells.

To further explore the expression pattern and determinants of *Ms4a3*, we compared the level of *Ms4a3* mRNA from freshly

isolated BM LSK cells to those from freshly isolated spleen LSK cells, as well as BM LSK cells cultured in vitro. In the freshly isolated spleen LSK cells from the control Grp94^{f/f} mice, the level of *Ms4a3* mRNA was 5% of the level detected in BM LSK cells (Fig. 4A). Similarly, a low level of *Ms4a3* mRNA was detected in the spleen LSK cells of the cGrp94^{f/f} mice. On examination of cultured BM LSK cells, a five-fold reduction in *Ms4a3* expression was observed in Grp94^{f/f} LSK cells cultured in vitro as compared with freshly isolated BM LSK cells. Thus, the expression of *Ms4a3* is context dependent, and the reduction of its expression on GRP94 depletion appears to be specific for freshly isolated BM LSK cells (Fig. 4A). In support of this, lentiviral shRNA-mediated GRP94 knockdown in the human promyelocytic leukemia cell line HL60, where MS4A3 is abundantly expressed, did not result in reduced *Ms4a3* expression (Fig. 4B, C). Collectively, these results support the notion of cell-type specificity and BM microenvironment dependency in the regulation of *Ms4a3* expression by GRP94.

GRP94 depletion reduced cell surface Cx32 plaque number but not CXCR4 and Tie2 expression

GRP94-null HSCs have impaired engraftment on transplantation, whereas transplanting Grp94^{f/f} hematopoietic

FIG. 3. Increased AKT activation is required for *cGrp94^{fl/fl}* LSK cell proliferation. **(A)** Fluorescence staining of phosphorylated-AKT (pAKT, Ser473; in green) on freshly isolated BM LSK cells from the indicated genotypes. The corresponding nuclei were stained by DAPI in blue. Scale bar represents 10 μ m. **(B)** Quantification of pAKT levels in *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* LSK cells with ImageJ (n = 41 for *Grp94^{fl/fl}*, n = 54 for *cGrp94^{fl/fl}*). *** P < 0.001. **(C)** Fluorescence staining of total AKT (in red) on freshly isolated BM LSK cells. **(D)** Quantification of total AKT levels in *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* LSK cells with ImageJ (n = 45 for *Grp94^{fl/fl}*, n = 64 for *cGrp94^{fl/fl}*). * P < 0.05. **(E)** Representative PI(3,4,5)P3 staining on freshly isolated BM LSK cells from *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* mice. The corresponding nuclei were stained by DAPI in blue. Scale bar represents 5 μ m. **(F)** Fold change in cell numbers from cultured LSK cells that were treated with either DMSO or MK2206 for 2 days at the indicated concentrations, with the number of cells seeded set as one. All data are presented as mean $\pm$ S.E. Color images available online at www.liebertpub.com/scd

cells into a GRP94-null microenvironment yielded a normal hematopoietic profile and HSC pool size [30]. Therefore, the molecules through which GRP94 regulates HSCs in their microenvironment are intrinsically expressed in HSCs but not niche cells. We examined potential cell surface proteins that maintain the HSC-niche interaction and the loss of which could affect HSC proliferation.

Gap junction protein Cx32 has been shown to be specifically expressed on the membrane of LSK cells, and the knockout of Cx32 leads to increased LSK cells in the BM through increased proliferation [26]. Despite the similar mRNA levels of Cx32 in the *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* LSK cells as determined by microarray analysis (*cGrp94^{fl/fl}*: *Grp94^{fl/fl}* = 1.07; Supplementary Table S1), a decreased Cx32 plaque number on the cell surface of freshly isolated GRP94-null LSK cells was observed when compared with the *Grp94^{fl/fl}* control (Fig. 5A). Quantitation of the images from confocal microscopy demonstrated that approximately 50% of the *Grp94^{fl/fl}* LSK cells had 5 or 6 plaques on the cell surface, with some cells

showing approximately 11 plaques (Fig. 5B). In contrast, 40% of GRP94-null LSK cells had no Cx32 plaques observed on the surface, and only a few cells showed five or more plaques (Fig. 5B). This indicates that the processing and formation of cell surface Cx32 plaques was disrupted on GRP94 ablation in LSK cells.

To determine whether the loss of cell surface Cx32 stimulates LSK cell proliferation, we utilized ³²Gap27, a Cx32 mimetic peptide designed to specifically block the Cx32 gap junction channel function via targeting its extracellular loop [32]. First, using a well-established scrape-loading and dye transfer assay in HeLa cells devoid of Cx32, we established that ³²Gap27, at a commonly used dosage (0.25 μ g/mL) [32,38,39], was able to inhibit Cx32-mediated gap junction function in HeLa cells when Cx32 was ectopically expressed (Fig. 6A and Supplementary Fig. S1). To test the effect on LSK cells, we isolated LSK cells from *Grp94^{fl/fl}* mice, which were equivalent to wild-type LSK cells due to the absence of the Cre-transgene, and the cells were either non-treated or

FIG. 4. Context-dependent expression of *Ms4a3* in LSK cells. **(A)** *Ms4a3* mRNA expression measured by quantitative real-time PCR on BM LSK cells, whole BM cells, Gr-1⁺, F4/80⁺ and B220⁺ BM cells, spleen LSK cells, and BM LSK cells cultured in vitro for 2 days. The levels of *Ms4a3* mRNA were normalized against 18S RNA. **(B)** Western blot analysis of GRP94 and β -actin expression in the human promyelocytic leukemia cells (HL60) infected with lentiviral shCtrl or shGrp94. **(C)** *Ms4a3* mRNA expression from HL60 cells infected with shCtrl and shGrp94 normalized against 18S RNA. The experiments were repeated two to four times. All data are presented as mean $\pm$ S.E., * P < 0.05, ** P < 0.01.

treated with scrambled peptides or ³²Gap27 at either 0.25 μ g/mL or a 10 times higher dose for 2 days before cell counting (Fig. 6B). We observed an increase in LSK cell number for non-treated and scrambled peptide-treated samples as expected; however, the number of cells from the ³²Gap27 groups were similar to the control groups at both peptide dosages (Fig. 6C). These data indicate that the loss of hemichannel function of cell surface Cx32 has no effect on LSK proliferation in vitro.

Meanwhile, two other important cell surface proteins maintaining HSC quiescence were not altered by GRP94 depletion. Despite the depletion of GRP94 in *cGrp94^{fl/fl}* LSK cells, as confirmed by western blot (Fig. 7A), cell surface expression of the angiopoietin receptor Tie2, which is required for maintaining the quiescence of HSCs, was not affected in GRP94-null LSK cells (Fig. 7B). At the mRNA level, comparable levels of Tie2 expression were observed in *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* LSK cells as revealed by microarray analysis (*cGrp94^{fl/fl}*: *Grp94^{fl/fl}* = 1.25; Supplementary Table S1). CXCR4 is a cell surface receptor that mediates the retention of HSCs in the BM niche, the knockout of which leads to increased HSC proliferation [18]. Recent studies also demonstrated the requirement of CXCR4 ligand CXCL12 in maintaining HSC population and function [17,40]. Microarray analysis revealed minimal effect on the mRNA levels of

CXCR4 in the LSK cells on *Grp94* knockout (*cGrp94^{fl/fl}*: *Grp94^{fl/fl}* = 0.75; Supplementary Table S1). Since the expression of mouse CXCR4 on LSK cells is too low to detect, possibly due to rapid turnover rate [41], we utilized the U266 human multiple myeloma cell as a model in which cell surface CXCR4 is abundant and detectable. In this model, knockdown of GRP94 expression mediated by shRNA (Fig. 7C) did not affect cell surface expression of CXCR4 (Fig. 7D). The lack of effect on CXCR4 expression on GRP94 depletion in the U266 cells is also consistent with that observed in GRP94-null mouse spleen B cells [42].

Discussion

GRP94, with its unique chaperone function, is emerging as an important player not only in protein processing but also in specific cellular processes that are distinct from other major chaperones in the ER. In particular, recent studies suggested that GRP94 is critically required for the functional expression of secretory and/or membrane proteins that enable the integration of cells into tissues [43]. We previously reported the role of GRP94 in maintaining the interaction between HSC and the adult BM niche using the *Grp94^{fl/fl}*; *Mx-1-Cre* mouse model. Inducible conditional loss of GRP94 in the hematopoietic system led to impaired HSC homing and engraftment, and an increase in proliferation that leads to an expansion of the GRP94-null HSC pool. In this report, we utilized additional criteria to establish the novel observation that GRP94 depletion has a major impact on HSC proliferation regulators, leading to the discovery of new mechanisms by which the lack of GRP94 can lead to loss of quiescence and LSK cell expansion in the BM.

We previously noted that using LSK cell markers in combination with Flk2 and CD34, both LT-HSC-enriched and ST-HSC-enriched populations were increased in the BM of the *cGrp94^{fl/fl}* mice [30]. Interestingly, in this study using the SLAM markers, while a two-fold increase of LSK cells and LT-HSC-enriched LSKCD41⁺ CD48⁺ CD150⁺ populations were observed in agreement with the earlier study, the ST-HSC-enriched LSKCD41⁺ CD48⁺ CD150⁺ cells remained comparable with those in the *Grp94^{fl/fl}* control. As inconsistency of changes in the LSKSLAM marker populations and LSKFlk2CD34 populations were also observed in other studies [44], the differences could be due to different coverage of cell populations identified by the two sets of markers. While the explanations for the observations that only LT-HSCs but not ST-HSCs are increased on *Grp94* deletion are not known, it is possible that the *cGrp94^{fl/fl}* LT-HSCs, while generating a larger population through increased proliferation, may be defective in differentiation into ST-HSCs. Alternatively, the *cGrp94^{fl/fl}* ST-HSCs may quickly differentiate into downstream cells, and, therefore, the reservoir of *cGrp94^{fl/fl}* ST-HSC remains similar compared with *Grp94^{fl/fl}* controls. In the *cGrp94^{fl/fl}* mice, the downstream lymphoid lineage differentiation is impaired [45], which could trigger a negative feedback signal to induce more differentiation from the ST-HSCs. Interestingly, immunofluorescent staining revealed that around 50% of *cGrp94^{fl/fl}* ST-HSCs displayed notably higher AKT activation when compared with the *Grp94^{fl/fl}* control (Supplementary Fig. S2).

How might increased HSC expansion be achieved on GRP94 deficiency? Here, we discovered that the loss of

FIG. 5. Reduction of Cx32 cell surface plaques on *Grp94* deletion. **(A)** Immunofluorescent Cx32 staining on freshly isolated *Grp94*^{fl/fl} and *cGrp94*^{fl/fl} BM LSK cells. White arrows indicate Cx32 plaques on the cell surface. Scale bar represents 2 μ m. **(B)** The number of Cx32 plaques was counted from Z-stack images for whole cells, and the percentages of *Grp94*^{fl/fl} (left) and *cGrp94*^{fl/fl} (right) BM LSK cells with different numbers of Cx32 plaques on the cell surface are presented. Color images available online at www.liebertpub.com/scd

quiescence in the *cGrp94*^{fl/fl} HSCs in the BM could be at least partially attributed to the increased activation of AKT. The increased proliferation and the elevated activation of AKT in GRP94-null LSK cells could be intrinsically restricted to primitive hematopoietic cells or a consequence of HSCs de-

taching from the BM endosteal niche and thereby escaping from the extrinsic cues maintaining their quiescence. Through culture of the isolated LSK cells in vitro in either the presence or absence of stromal cells, we validated that loss of GRP94 in BM LSK cells leads to a significant increased cell

FIG. 6. Effect of Cx32 mimetic peptides on LSK cell proliferation in vitro. **(A)** Validation of the ability of ³²Gap27 in blocking gap-junctional intercellular communication (GJIC) by scrape-loading and dye transfer assay. HeLa cells transfected with empty vector or Cx32 expression vector were scraped and loaded with the mixture of Lucifer yellow and Rhodamine dextran dyes. The level of intracellular communication, as indicated by the ratio between the cells receiving Lucifer yellow from neighboring cells and the cells initially labeled under various experimental conditions, is shown. **(B)** Scheme of experimental design for treatment and assay with mimetic (³²Gap27) or scrambled peptides. **(C)** Cell numbers from the cultures subjected to the different treatment conditions as indicated next. The concentrations of the peptides in μ g/mL are shown. NT, not treated. All data are presented as mean $\pm$ S.E.

FIG. 7. Lack of effect of GRP94 depletion on Tie2 and CXCR4 surface expression. **(A)** Western blot analysis of GRP94 expression in the *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* BM, with β -actin serving as loading control. **(B)** Cell surface Tie2 expression on *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* BM LSK cells determined by flow cytometric analysis. The green line represents *Grp94^{fl/fl}* cells, and the red line indicates *cGrp94^{fl/fl}* cells. **(C)** GRP94 expression in the U266 multiple myeloma cells infected with lentiviral shCtrl or shGrp94 determined by western blot. **(D)** Representative flow cytometric analysis on cell surface CXCR4 expression on the U266 cells. The gray area represents isotype control staining; the blue line indicates NT cells; the green line represents U266 cells infected with shCtrl; and the red line indicates U266 cells infected with shGrp94. Color images available online at www.liebertpub.com/scd

expansion. Similarly, this increase in cell expansion could be a cell intrinsic property of GRP94-null HSCs in vivo. However, we cannot rule out the possibility that the defective niche retention of the GRP94-null HSCs in vivo alters LSK cell physiology and leads to cell expansion both in vivo and in vitro. We previously identified that GRP94 deficiency leads to loss of cell surface integrin $\alpha 4$ despite no effect on its mRNA level (*cGrp94^{fl/fl}*: *Grp94^{fl/fl}* = 1.11; Supplementary Table S1) [30]. The loss of cell surface integrin $\alpha 4$ could be partially responsible for the increased AKT activation. It has been shown that the binding of integrin $\alpha 4$ on the erythroid progenitor cells with fibronectin reduced AKT activation [46]. This is consistent with our phenotype, in which the loss of integrin and its loss of binding to fibronectin could release the inhibition of AKT activation on HSC. However, the loss of integrin $\alpha 4$ alone is not sufficient to cause the hyperproliferation phenotype, as analysis of integrin $\alpha 4^{-/-}$ mice or conditional deletion of integrin $\alpha 4$ did not show increased LSK cell number or proliferation [23,47]. Therefore, there may be other factors influencing AKT signaling and the hyperproliferation of HSC. The mild reduction of total AKT on GRP94 deficiency may have resulted from the post-transcriptional regulation of AKT by caspase cleavage [48], as GRP94 depletion has been reported to trigger mild caspase activation [49]. Reduction of total AKT level has also been reported in other studies following various treatments [50,51], and other regulatory factors may be involved, which awaits future investigation.

Interestingly, the effect of ER chaperone deficiency on the activation of AKT may not only be context dependent but also chaperone specific. Deficiency of another major ER

chaperone, GRP78, impairs the activation of PI3K-AKT pathway in vitro as well as in PTEN-null prostate cancer and leukemia mouse models [36,52]. This reflects the distinct function of individual ER chaperones besides their common capacity of assisting protein folding, and this could be attributed to their unique localization, characteristics of client proteins, and other possible functions such as mediating signal transduction. While the mechanisms by which GRP94 deficiency promotes PI(3,4,5)P₃ production and AKT activation remains to be determined, it is noted that as in the case of several other ER chaperones, GRP94 can also be localized on the cell surface of specific cell types [53]. It is tempting to speculate that cell surface GRP94 could act as a suppressor for PI3K signaling and its ablation will relieve the inhibition, resulting in increase of PI(3,4,5)P₃ production and AKT activation.

Through microarray analysis, we explored how GRP94 depletion alters the gene expression profile of LSK cells, which shows a dramatic down-regulation of *Ms4a3*, a negative cell cycle regulator in the context of hematopoietic cells. Human MS4A3 is reported to bind to cyclin-dependent kinase-associated phosphatase-CDK2 (KAP-CDK2) complexes, stimulates the phosphatase activity of KAP, and thereby regulates cell cycle progression [8,10]. Unfortunately, since no antibody against mouse MS4A3 is available, our studies have been restricted to measurement of its transcript level. However, its expression pattern in freshly isolated LSK cells from BM and spleen and after LSK cell culture in vitro reveal new information. Interestingly, the reduction in *Ms4a3* expression is restricted in freshly isolated GRP94-null LSK cells, whereas its expression in the GRP94-null BM is moderately increased when compared with the wild-type control. This increased *Ms4a3* expression could be attributed to its increased expression in the Gr-1⁺ cells and F4/80⁺ cells, the two of which together comprise more than 50% of BM cells. Compared with wild-type BM LSK cells, *Ms4a3* expression is dramatically reduced in spleen LSK cells. As a negative regulator of the cell cycle, the much lower *Ms4a3* expression in the spleen LSK cells in comparison with the BM LSK cells is also consistent with their proliferative status, as spleen HSCs cycle twice as frequently as do BM HSCs [54]. While the *Ms4a3* expression is similarly low in *Grp94^{fl/fl}* and *cGrp94^{fl/fl}* spleen LSK cells, we previously reported that the number of LSK cells actually increased by 2.5-fold in the *cGrp94^{fl/fl}* spleen when compared with the control, likely resulting from increased HSC mobilization on GRP94 depletion [30]. Supporting this notion was the loss of LSK cell surface integrin $\alpha 4$ expression on GRP94 depletion and a seventeen-fold increase in circulating *cGrp94^{fl/fl}* LSK cells in the peripheral blood [30]. The low expression of *Ms4a3* in the spleen LSK cells and cultured BM LSK cells is consistent with a possible role of BM microenvironment in regulating the expression of *Ms4a3*. Evidence indicates that environmental components in the BM niche may regulate the expression of *Ms4a3*, as we observed a high level of *Ms4a3* mRNA expression only in freshly isolated *Grp94^{fl/fl}* BM LSK cells but not in *cGrp94^{fl/fl}* BM LSK cells, nor in freshly isolated spleen LSK cells of either genotypes, or BM LSK cells cultured in vitro, in all of which cases there is no intact HSC-BM niche interaction. The HSC niche in the splenic microenvironment may comprise different components from the BM HSC niche [55] due to the lack of osteoblastic cells and trabecular bone. In addition,

different subtypes of mesenchymal stem cells or endothelial cells may comprise the HSC niche in the spleen [14–16,55], which may contribute to the differential expression level of *Ms4a3* in the BM and spleen. Thus, loss of GRP94 in the LSK cells and the subsequent loss of niche attachment could trigger the down-regulation of *Ms4a3*, contributing to loss of quiescence and cell expansion. Unfortunately, we are unable to test the functional contribution of *MS4A3* in GRP94-null mediated LSK cell expansion in vitro by knockdown, as the differential levels of *Ms4a3* between the two genotypes only occurred in vivo and the expression of *Ms4a3* was lost when LSK cells were cultured in vitro.

Our studies also revealed that GRP94-null HSCs has impaired cell surface Cx32 plaque formation, and Cx32-knockout mice exhibited a loss of quiescence and hyperproliferation of HSCs [26]. However, this reduction in Cx32 plaque number on GRP94-null HSCs is likely due to defective processing in the ER, as microarray analysis on freshly isolated *Grp94^{ff}* and *cGrp94^{ff}* LSK cells revealed a comparable level of Cx32 mRNA. While it will not be possible to rescue cell surface Cx32 expression by ectopic expression in GRP94 null LSK cells with intrinsic protein processing defects, we were able to test whether Cx32 gap junction function affects LSK cell proliferation in vitro. Our studies blocking Cx32 hemichannel function with a Cx32 mimetic peptide did not result in increased proliferation. There could be several explanations for this. First, the increased Cx32-null LSK cell proliferation in the mouse model could be due to the complete depletion of Cx32 by genetic knockout, whereas blocking the function of membrane Cx32 may only partially suppress its activity such that it is not sufficient to alter LSK cell proliferation. Second, the regulation of HSC proliferation by surface Cx32 plaques may be independent from the function of Cx32 as a gap junction protein, and, therefore, targeting the hemichannel function of Cx32 with mimetic peptides will not be effective. Third, it is also possible that the reduced surface Cx32 plaques on GRP94 depletion is not a cause but a consequence of increased proliferative signals in *cGrp94^{ff}* LSK cells, such as elevated AKT phosphorylation. In support of this, constitutively activated AKT has previously been shown to suppress Cx32 mRNA and protein levels [56]. We also examined the expression of Cx43 and Cx26 by immunofluorescent staining; however, their levels were below detection limit in the LSK cells in our experimental system (data not shown). Interestingly, two other major cell surface receptors linked to stem cell quiescence, Tie2 and CXCR4 expression, were not impaired on GRP94 depletion in hematopoietic cells.

In summary, our studies provide the first evidence that GRP94 deficiency leads to AKT activation, suppresses *Ms4a3* expression, and impairs Cx32 plaque formation in BM LSK cells, factors that have been linked to stem cell proliferation. Studies with inhibitors of AKT and Cx32 also provide mechanistic insights on their requirement in mediating the hyperproliferation resulting from GRP94 depletion in the HSCs. Our studies demonstrate that GRP94, which is traditionally regarded as an ER chaperone protein with a primary function of protein folding and assembly, has novel roles in regulating HSC proliferation, growth signaling, and cell cycle regulators. The diverse role of ER chaperones reflects the complex nature by which chaperone proteins regulating cellular processes in different contexts, both intrinsically and

in interacting with the microenvironment, warrant future investigation.

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Authors Disclosure Statement

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Address correspondence to:

Dr. Amy S. Lee

Department of Biochemistry and Molecular Biology

USC Norris Comprehensive Cancer Center

Keck School of Medicine

University of Southern California

1441 Eastlake Avenue

Los Angeles, CA 90089-9176

E-mail: amylee@ccnt.usc.edu

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