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Mesenchymal stem cells in ribosomal insufficiency lead to aberrant osteoblastic differentiation and altered hematopoiesis

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Running heads: Defective BM MSC of DBA

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Authorship contributions:

H.N.K., J.Y.W., and K.M.S conceptualized and designed the study. A.S., K.M., and Y.L.L. provided Cre-ERT2 and MX1-Cre Rpl11 mouse models for the experiment. D.C.S., L.V., A.G., B.E.G., and K.W provided healthy and patient samples. F.S. and H.N. contributed to generating healthy donor- and DBA-patient-derived induced pluripotent stem cells (iPSCs). D.F analyzed directional differential gene expression from RNA-sequencing data. J.V contributed to cell proliferation assays. H.N.K. designed and performed the experiments, analyzed and visualized the data. H.N.K wrote the original draft. All the authors reviewed and contributed to editing the manuscript.

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Abstract

Diamond-Blackfan anemia (DBA) is an inherited bone marrow failure syndrome (IBMFS) characterized by macrocytic anemia. In addition to anemia, patients present with skeletal defects and an increased risk of osteosarcoma, suggesting dysregulated bone cell development. We found that ribosomal protein large subunit 11 (RPL11) haploinsufficiency affects bone marrow mesenchymal stem cells (MSCs), which are the precursors of osteoblasts that support lifelong hematopoiesis. Mouse and human DBA MSCs show decreased proliferation due to cytokinesis failure. Bulk RNA sequencing identified that DBA MSCs are enriched in gene sets associated with inflammation and bone remodeling that can affect surrounding hematopoietic cells. RNA sequencing further predicted aberrant bone maturation in DBA MSCs due to downregulation of the YAP pathway. *In vitro* differentiation assay confirmed that both mouse and human DBA MSCs fail to differentiate into osteoblasts compared with control MSCs. Activation of YAP signaling improved cell proliferation, morphology, and osteoblast differentiation potential in DBA MSCs. Lastly, we identified that DBA MSCs reduce the expansion of healthy hematopoietic stem and progenitor cells (HSPCs) through osteopontin. In contrast, DBA MSCs provide a favorable niche for DBA HSPCs and significantly increase the expansion of multipotent progenitors (MPPs). However, these MPPs have limited differentiation potential and do not mature into subsequent committed progenitors in the bone marrow. Taken together, this study provides an overview of the defective functions of DBA MSCs, informing follow-up studies to advance our understanding of niche-mediated pathogenic hematopoiesis of DBA.

Introduction

Diamond-Blackfan anemia (DBA) is an inherited bone marrow failure syndrome (IBMFS) characterized by macrocytic anemia. More than half of patients carry heterozygous mutations in ribosomal protein (RP) genes,^{1,2} including *RPS19*, *RPL5*, *RPL11*, *RPL26*, and *RPL10*. Non-RP mutations (*GATA1*,^{3,4} *TSR2*,⁵ *TP53*,^{6,7} and *HEATR3*⁸) perturbing erythropoiesis also cause “DBA-like” symptoms. In addition to anemia, patients present with triphalangeal thumbs, cleft palate, short stature,^{1,9} and an increased risk of osteosarcoma,¹⁰ indicating overall dysfunctional bone development within the bone marrow microenvironment (BME).

In DBA, p53 upregulation through the RP-HDM2 (MDM2 in mice)-p53 axis has been identified as a key underlying mechanism of anemia.^{11,12} However, normal p53 expression and p53-independent mechanisms are reported,^{13,14} suggesting that additional mechanisms contribute to the disease’s pathogenesis. More recently, emerging evidence suggests that an aberrant bone marrow microenvironment (BME) contributes to the phenotypes of inherited bone marrow failure syndromes (IBMFS).¹⁵ Yet, current DBA therapies and research have primarily focused on the hematologic compartment, leaving the defective BME and its pathological mechanisms poorly understood. In this study, we characterized mesenchymal stem cells (MSCs), osteoblast precursors¹⁶ that support lifelong hematopoiesis by maintaining hematopoietic stem and progenitor cells (HSPCs)^{17,18}, to understand their contribution to the disease’s skeletal phenotypes and anemia using mouse and human DBA MSCs with ribosomal protein large subunit 11 (*RPL11*) haploinsufficiency.

Methods

Use of human and mouse-derived cells for the study.

Use of human and mouse samples is approved by Institutional Review Board (IRB) protocols. Human bone marrow aspirates and biopsies are acquired under IRB protocol with informed consent. Use of human-derived induced pluripotent stem cells (iPSCs) is approved under Stem Cell Research Oversight (SCRO) protocol. All mice used in this study were maintained in accordance with an

approved Institutional Animal Care and Use Committee (IACUC) protocol and regulations to ensure ethical animal care.

Mouse and human MSC isolation and ex vivo culture.

Flushed mouse cells were RBC-lysed and cultured in MSC medium (MesenCult; Stemcell Technologies) with 1% penicillin-streptomycin (P/S) in a 12-well tissue culture plate (1×10^6 /mL). For human MSCs, bone marrow biopsies were cut into small pieces and cultured in MSC medium. After 48 hours, the medium was replenished every other day to deplete hematopoietic cells until adherent cells reached confluence. Expanded cells were trypsinized with 0.05% trypsin-EDTA to characterize MSC surface markers and tri-lineage differentiation potential. For *in vitro* deletion of *Rpl11*, WT MSCs were treated with cell-permeable Cre (Cre Recombinase Gesicles, Takara) following the manufacturer's protocol. For proliferation assays, 1×10^4 of mice or 2×10^4 human MSCs were seeded onto a 12-well tissue culture plate, and drugs were added after 24 hours if needed. The growth medium was replenished every other day until MSCs were trypsinized and harvested for cell counting. Cell counts were assessed using Trypan blue staining.

Osteoblast differentiation of MSCs

1×10^4 of MSCs were seeded onto a 24-well tissue culture plate in 0.5mL of MSC medium. After 24 hours, cells were washed with DPBS and replenished with 0.5mL of osteoblast differentiation medium (DMEM, 10% FBS, 50 μ g/mL ascorbic acid, 10mM β -glycerol phosphate, and 100nM dexamethasone). Medium was changed every 3-4 days for 4 weeks. Differentiated cells were stained with Alizarin Red (Lifeline Cell Technology) to visualize the calcium crystals produced by osteoblasts following the reagent's protocol.

Co-culture of HSPCs with MSCs and flow cytometry

1x10⁴ of MSCs were seeded onto a 6-well tissue culture plate. After 24 hours, 5x10⁴ of total mouse BM, enriched HSPCs (MojoSort™ Mouse Hematopoietic Progenitor Cell Isolation Kit (Biolegend) or human bone marrow CD34⁺ cells (StemCell Technology) were added onto MSC-coated plates. The co-culture medium is 1:1 of low-glucose DMEM:IMDM supplemented with 10% FBS, 1 ng/mL of SCF, TPO, FLT3L, IL-3, and IL-6, and 1% P/S. To assess erythropoiesis on MSCs, 3U/mL of erythropoietin was added to the medium. For flow cytometry, hematopoietic cells were harvested by vigorous pipetting and collected through a 40 µm cell strainer. HSPCs were washed and stained with flow cytometry antibodies. In a panel including CD34, HSPCs were first stained with an anti-CD34 antibody for 30 minutes, then with the remaining antibodies for an additional 30 minutes. After staining, cells were washed and analyzed on a Quanteon Flow Cytometer (Agilent Technologies). A list of antibodies is provided in Supplementary Table 2.

Statistical analysis

We performed statistical analyses using GraphPad Prism and present results as mean ± standard deviation (SD). To compare means between two groups, we used a Student's t-test. For comparisons of more than two outcomes, we used two-way ANOVA with a multiple comparisons test. A p-value or adjusted p-value of less than 0.05 is considered statistically significant.

Additional materials and methods are provided in the supplementary data.

Results

Mouse and human DBA MSCs proliferate more slowly compared to control MSCs.

To characterize RPL11 haploinsufficiency in MSCs, we first isolated MSCs from Cre-ERT2-Rpl11^{+/-lox} ¹⁹ wild-type (WT) and DBA mice (DBA) (Supplementary Figure S1A-C) by the lymphodepletion method (Supplementary Figure S1D-G). DBA MSCs show rounded morphology (Figure 1A) and proliferate more slowly than WT MSCs (7.17x10⁴±0.45 (WT) and 3.24x10⁴±0.35 (DBA); p<0.0001,

n=5) (Figure 1B). Mx-Cre-Rpl11^{+/-lox}-derived DBA MSCs²⁰ also showed decreased proliferation compared with WT MSCs ($6.87 \times 10^4 \pm 1.55$ (WT) and $4.09 \times 10^4 \pm 1.41$ (DBA); $p=0.001$, $n=4$) (Figure 1B). To further rule out the effect of long-term inflammation on reduced proliferation, we deleted Rpl11 *Ex vivo*. We observed the same proliferation reduction in Rpl11-deleted MSCs ($6.21 \times 10^4 \pm 1.68$ (-Cre) controls and $1.70 \times 10^4 \pm 1.56$ in (+Cre Rpl11^{+/-Δ}); $p=0.0027$, $n=3$) (Figure 1B), indicating that the proliferation defect is directly associated with RPL11 haploinsufficiency, irrespective of mouse strain. Reduced carboxyfluorescein succinimidyl ester (CFSE) and Ki-67 expression further support the slow proliferation of DBA MSCs compared to WT MSCs (Figure 1C). In DBA MSCs, G2/M phase was significantly increased compared to WT ($17.6\% \pm 1.87$ (WT) and $37.9\% \pm 6.4$ (DBA); $p=0.0062$, $n=3$) (Figure 1D). Synchronization culture of MSCs after serum starvation shows that the G2/M phase increase is due to failure to exit the G2/M phase and re-enter the cell cycle (Supplementary Figure S2A-C). G2/M phase-specific mRNA (Figure 1E) and proteins (Figure 1F) cyclin A, cyclin B, and CDK1 were decreased in DBA MSCs, while other cell cycle markers did not change (Supplementary Figure S2D). Interestingly, p53 mRNA and protein expression were downregulated in DBA MSCs (Supplementary Figure S2E) compared to control, which contrasts with the elevated p53 observed in DBA erythroid progenitors.^{21,22,12} Despite arrest in the cell cycle, DBA MSCs did not undergo apoptosis (Supplementary Figure S2F), suggesting potential activation of pro-survival pathways. To validate these results in human MSCs, RPL11 haploinsufficient human DBA MSCs were generated using CRISPR/Cas9 knockout (KO) (Figure 2A) and induced pluripotent stem cell (iPSC) methods (Figure 2C, Supplementary Figure S3A-E). Real-time quantitative PCR (qPCR) confirmed a significant reduction in *RPL11* mRNA in KO (1.010 ± 0.17 in negative control (NC) and 0.78 ± 0.094 in KO; $p=0.018$, $n=4$) (Figure 2C) and iPSC-derived MSCs (iMSCs) (1.013 ± 0.19 (HD iMSCs) and 0.4925 ± 0.072 (DBA patient iMSCs); $p=0.0008$, $n=4$) (Figure 2D) compared to controls. As in mice, human DBA MSCs were rounder than healthy controls (Figure 2A, B). In addition, both RPL11 KO (Figure 2E) and DBA iMSCs (Figure 2F) grew more slowly than their controls, without reduced cell viability, consistent with DBA mouse MSCs. We also isolated MSCs from a healthy donor (HD) and

from a DBA-like patient BM biopsy (*ASXL* and *PLCB1* mutations) and observed reduced proliferation (Supplementary Figure S3F).

Characterization of transcriptomic profile of DBA MSCs before and after osteoblastic differentiation using bulk RNA-sequencing.

To identify differentially expressed disease-associated genes in DBA MSCs, we performed bulk RNA sequencing (RNA-seq) using mouse MSCs before and after osteoblast differentiation (Figure 3A). PCA plots show tight clustering of replicates between WT and DBA MSCs at each time point (Figure 3B). UpSet plots showed significant overlap of differentially expressed genes between the 2-week- and 4-week-differentiated MSCs, suggesting that maximal genetic change during osteoblast differentiation occurs within the first 2 weeks and persists (Figure 3C). Using the differentially expressed gene (DEG) list for each time point, we identified pathways enriched for the defined gene sets from the Kyoto Encyclopedia of Genes and Genomes (KEGG). In undifferentiated DBA MSCs, genes mediating extrinsic signals (extracellular matrix proteins (ECM), cell adhesion, and cytokine interactions) are enriched compared to WT MSCs (Figure 3D). Genes in TGF- β signaling and PI3K/AKT pathway, which were previously reported to be dysregulated in DBA hematopoietic cells,^{19,23,24} were also differentially enriched in DBA MSCs. These pathways shared a list of genes, such as bone morphogenic proteins (*Bmp2*, *Bmp7*, and *Bmp4*), integrins (*Itga1*, *Itga2*, *Itga3*, *Itga6*, *Itgb1*, and *Itga2*), and ECM components (laminin, collagen, and osteopontin), indicating that differential expression of the same gene sets contributes to multiple signaling pathways in response to RPL11 haploinsufficiency. Ingenuity Pathway Analysis (IPA) using DEGs predicted enrichment for bone differentiation, cytokine-mediated hematopoietic cell responses, and cancer progression in DBA MSCs (Figure 3D), based on the high expression of *Bmp2* in DBA MSCs. However, elevated *Bmp2* did not lead to increased BMP2 protein expression in DBA MSCs (Supplementary Figure S4A), suggesting that these predicted pathways may not be functionally active in DBA MSCs.

After 2 weeks of osteoblast differentiation, DBA MSCs differentially expressed genes that are involved in osteoblast-regulating pathways (Wnt,²⁵ JAK-STAT pathways²⁶) compared to WT MSCs (Figure 3E). Gene sets mediating extrinsic signals (ECM, cell adhesion, and cytokine interactions) and the PI3K-AKT signaling pathway remain differentially enriched in DBA MSCs as before (Figure 3D), suggesting differential expression of genes mediating these pathways may affect overall MSC functions irrespective of maturation stages. IPA analysis predicted that downregulation of TEAD2 and TP53 activity, both of which are known to regulate osteogenesis,^{27,28} contributes to decreased tyrosine phosphorylation of proteins, cell motility, connective tissue formation, and other processes in DBA MSCs (Figure 3D). At the end of 4 weeks of osteoblast differentiation, DBA MSCs maintained similar gene signatures compared to 2 weeks of differentiation, as expected with the UpSet plot (Figure 3C). However, more bone development-associated pathways (Wnt, cAMP,²⁹ and MAPK³⁰ pathways) are differentially enriched than in 2 weeks of differentiation (Supplementary Figure S4B). These genetic signatures further contributed to the downregulation of proliferation, tyrosine phosphorylation, differentiation, and bone mineralization (Supplementary Figure S4B) in DBA cells. Overall, our RNA sequencing analysis suggests that *RPL11* haploinsufficiency leads to differential enrichment of genes that dysregulate primary signaling pathways and influences broad metabolic and biologic pathway reprogramming during osteoblast differentiation.

Cytokinesis failure is associated G2/M phase arrest in DBA MSCs.

As described earlier, DBA MSCs were rounder (Figure 1A; Figure 2A, B) and grew more slowly than control MSCs (Figure 1B; Figure 2E, F). To further understand morphological changes and impaired proliferation, we first stained mouse MSCs with antibodies against cytoskeletal proteins and RPL11. Immunofluorescence images show perinuclear localization of RPL11 and increased accumulation of F-actin beneath the plasma membrane in DBA MSCs compared to WT MSCs (Figure 4A, Supplementary Figure S5A). Other major types of cytoskeletons, α -tubulin and Lamin A/C, did not show a significant change (Supplementary Figure S5B, C) compared to F-actin. Interestingly,

binucleation was significantly increased in DBA MSCs compared to WT MSCs ($4.995\% \pm 1.596$ (WT) and $27.01\% \pm 7.501$ (DBA); $p=0.0007$, $n=4$ for WT and $n=5$ for DBA. Each image contains >50 nucleated cells.) (Figure 4B). Given G2/M-phase arrest in DBA MSCs, we hypothesized that impaired cytoskeletal organization is associated with cytokinesis failure, leading to multinucleation. Time-lapse imaging of live mouse MSCs showed cleavage furrow formation followed by the separation into two daughter cells in WT MSCs (Figure 4C, top row, white arrow) during mitosis. In contrast, the cleavage furrow did not progress (Figure 4C, bottom row, white arrow) in nearly 80% of the total mitotic events in DBA MSCs ($0\% \pm 0$ (WT) and $86.67\% \pm 23.09$ (DBA); $p=0.0229$, $n=4$ for WT and $n=3$ for DBA. Each imaging includes >4 mitotic events.) (Figure 4D), resulting in multinucleation in a cell. From yeast to mammalian cells, cleavage furrow progression is mediated by actomyosin contraction,^{31,32} which is primarily composed of myosin-II molecules and actin filaments.^{33,34} Although it was not among the top pathways, we found that the cytoskeleton regulation pathway is differentially enriched in DBA MSCs with statistical significance (Supplementary Figure S5D). This gene set included two major non-muscle myosin class II protein genes, *Myh9* and *Myh10* (Supplementary Figure S5D). Despite decreased mRNA levels, *Myh9* protein was similarly expressed in DBA as in WT (Figure 4E). In contrast, *Myh10* protein expression was significantly reduced in DBA MSCs compared to WT (Figure 4E), suggesting *Myh10*-mediated cytokinesis contraction may be abnormal in DBA MSCs.^{35,36} DBA MSCs also downregulated *RhoA* expression, a master regulator of contractile ring formation and activation, indicating that these cells display reduced potential for cellular contraction during cytokinesis. Immunofluorescent imaging of dividing cells shows the formation of a thick actin plate that is devoid of RPL11 protein in the cleavage furrow regions of DBA MSCs (Supplementary Figure S5E), suggesting a potential role of RPL11 in cytokinesis. These data suggest that impaired cleavage furrow progression due to aberrant actin organization and *Myh10* deficiency is associated with cytokinesis failure in DBA MSCs.

Inactive YAP is associated with defects in proliferation and osteoblast differentiation in DBA MSCs.

Recently, cytokinesis failure has been found to inactivate yes-associated protein (YAP) to restrict the proliferation of genetically unstable cells.⁴⁰ Our RNA sequencing analysis also predicted that decreased TEAD2, a major binding partner of YAP, as one of the top regulators resulting in inactivation of downstream pathways (Figure 3E). In MSCs, active YAP migrates into the nucleus and binds to TEAD to promote cell proliferation, survival, and osteoblast differentiation.^{41,42} We found that YAP localizes primarily in the cytoplasm in mouse and human DBA MSCs, whereas it localizes in the nucleus in control MSCs (Figure 5A). Western blot further showed increased phosphorylation at serine 127 and total cytoplasmic YAP in mouse DBA MSCs (Figure 5B), indicating inactive YAP sequestered in the cytoplasm. We then investigated if impaired YAP is associated with osteoblast differentiation in DBA MSCs. After 4 weeks of osteoblast differentiation, WT MSCs' morphology changed uniformly (Supplementary Figure S6A). In contrast, DBA MSCs were heterogeneous in morphology, including multinucleated round cells and spindle-shaped cells (Supplementary Figure S6A). Alizarin red staining shows a significant reduction in osteoblast differentiation in both mouse and human DBA MSCs compared to control MSCs. (Figure 5C, left column). More strikingly, osteoblast differentiation led to apoptosis of human DBA MSCs, resulting in no visible viable cells with alizarin red staining (Figure 5C, right column). Osteogenic markers RUNX2 and β -catenin were also significantly downregulated in DBA MSCs compared to WT MSCs (Figure 5D), indicating inefficient osteoblast maturation. We next investigated whether restoring YAP activity could reverse the phenotypic defects of DBA MSCs. We first treated mouse WT and DBA MSCs with YAP activator TRULI at different concentrations and found that 1 μ M TRULI significantly increased WT and DBA MSC expansion ($1.22 \times 10^4 \pm 0.1$ (WT+DMSO) and $2.06 \times 10^4 \pm 0.02$ (WT+TRULI); $p < 0.0001$, $n = 6$. $0.46 \times 10^4 \pm 0.02$ (DBA+DMSO) and $0.88 \times 10^4 \pm 0.1$ (DBA+TRULI); $p < 0.0001$, $n = 6$) (Figure 5E) and stellate morphology (Supplementary Figure S6B) after 7 days of culture. Most importantly, TRULI treatment increased alizarin red staining in DBA MSCs, indicating that YAP restoration alleviates the proliferation and osteoblast differentiation defects in DBA MSCs (Figure 5F). Although YAP activation is not specific to DBA MSCs, TRULI was the only treatment that DBA MSCs responded to among

other drugs tested, including corticosteroids and L-leucine (Supplementary Figure S6C-E). These results demonstrate that impaired nuclear localization of YAP proteins leads to morphologic changes, impaired growth, and reduced osteoblast differentiation in DBA MSCs, and that restoring YAP signaling can improve these phenotypic defects.

DBA MSCs suppress the expansion of hematopoietic stem and progenitor cells (HSPCs) through osteopontin.

MSCs play a critical role in maintaining hematopoietic stem and progenitor cells (HSPCs) as a part of the BM niche. As a result of RPL11 haploinsufficiency, our RNA sequencing analysis predicted dysregulated interactions between defective DBA MSCs and hematopoietic cells (Figure 3D). To investigate the functional effect of DBA MSCs on hematopoiesis, we first determined if DBA MSCs affect the expansion of healthy HSPCs. We co-cultured healthy mouse and human HSPCs with DBA and control MSCs. After 7 days, both total BM ($4.3546 \times 10^5 \pm 0.165$ (WT MSC) and $1.2008 \times 10^5 \pm 0.294$ (DBA MSCs; $p=0.0025$) (Supplementary Figure S7A) and lineage-negative (Lin⁻) HSPCs ($1.192 \times 10^5 \pm 0.514$ (WT MSC) and $0.608 \times 10^5 \pm 0.133$ (DBA MSCs; $p=0.00019$) (Figure 6A, left column) decreased significantly in the presence of DBA MSCs. Human BM CD34⁺ cells cultured on DBA iMSCs also decreased in cell numbers compared to HD iMSCs ($6.898 \times 10^4 \pm 0.344$ (HD iMSC) and $5.062 \times 10^4 \pm 0.7689$ (DBA iMSCs); $p=0.003$, $n=5$) (Figure 6A, right column), suggesting DBA MSCs regulate HSPC expansion. To further identify potential targets affecting HSPC proliferation, we screened differentially expressed extrinsic factors using an antibody array. Among 96 analytes, we identified six differentially expressed cytokines in DBA MSCs (Figure 6B, left column, Supplementary Figure S7B), with osteopontin showing the greatest fold increase consistent with RNA-seq. ELISA assay validated increased osteopontin levels in both mouse (1095 ± 620.0 pg/mL (WT) and 2992 ± 855.9 pg/mL (DBA); $p<0.0001$, $n=11$; Supplementary Figure S7C) and human (16.53 ± 2.664 ng/mL for (HD iMSC) and 26.14 ± 3.026 ng/mL for (DBA iMSCs); $p=0.0145$, $n=3$. 2.550 ± 0.08 ng/mL (HD) and 18.19 ± 3.846 ng/mL (DBA patient MSCs); $p=0.0289$, $n=2$; Supplementary

Figure S7D) DBA MSCs compared to controls. Western blot shows increased cleaved osteopontin in DBA MSCs (Figure 6B, right column), the active form of the protein. In the normal BM, osteopontin is primarily produced by osteoblasts, and the gradient is high in the endosteal region where long-term HSC (LT-HSC) quiescence and self-renewal are maintained.^{17,43} To determine if reduced proliferation is mediated by osteopontin produced by DBA MSCs, we treated mouse and human HSPCs with osteopontin (Figure 6C). As a result, osteopontin reduced the expansion of both mouse ($2.42 \times 10^4 \pm 0.76$ (1% BSA) and $1.12 \times 10^4 \pm 0.50$ (50ng/mL OPN); $p=0.0377$, $n=8$ for 1% BSA and $n=7$ for OPN) (Figure 6D, left column) and human HSPCs ($3.725 \times 10^4 \pm 0.4232$ (1% BSA) and $2.7883 \times 10^4 \pm 0.4653$ (50ng/mL OPN); $p=0.0045$, $n=6$) (Figure 6D, right column) compared to their controls. We further tested whether osteopontin inhibition can restore suppressed HSPC expansion in DBA MSCs using an osteopontin-specific antibody (Figure 6E). After 7 days, anti-osteopontin antibody treatment increased HSPC expansion on DBA MSCs in a dose-dependent manner (Isotype vs 10ng/mL $p=0.036$; Isotype vs 100ng/mL $p=0.0025$; Isotype vs 1 μ g/mL $p=0.0224$ on day 7) (Figure 6F), while not affecting HSPCs on WT MSCs. These data suggest that osteopontin overexpression by DBA MSCs suppresses the expansion of healthy HSPCs.

DBA MSCs selectively accumulate multipotent progenitors with low differentiation potential.

Lastly, we investigated the effect of DBA MSCs on DBA HSPCs. To determine this, we cultured WT and DBA HSPCs on WT and DBA MSCs. Consistent with the previous results, WT HSPCs expansion was significantly reduced on DBA MSCs (Figure 7A, left column). In contrast, DBA HSPCs expansion was significantly increased in the presence of DBA MSCs compared to WT MSCs (Figure 7A, right column), suggesting that DBA MSCs provide a more suitable niche for DBA HSPCs survival and growth. Microscopic images showed HSPC expansion on MSCs consistent with the proliferation results (Supplementary Figure S8A). To identify the hematopoietic trajectory, we stained WT and DBA HSPCs cultured on MSCs with hematopoietic stem and progenitor cell antibodies and analyzed them by flow cytometry. As a result, we found that DBA MSCs significantly increase the lineage-

negative (Lin⁻) fraction of both WT and DBA HSPCs compared to WT MSCs (WT HSPC + WT MSC vs WT HSPC + DBA MSC: $p=0.0025$, $n=5$. DBA HSPC + WT MSC vs DBA HSPC + DBA MSC: $p=0.0014$, $n=5$) (Figure 7B, Supplementary Figure S8B). Within this population, LSK (Lin⁻cKIT⁺Sca-1⁺) was enriched in both WT and DBA HSPCs (WT HSPC + WT MSC vs WT HSPC + DBA MSCs: $p=0.0106$, $n=4$. DBA HSPC + WT MSC vs DBA HSPC + DBA MSC: $p=0.0003$, $n=4$) (Figure 7C, D). This increase was due to an increased percentage of multipotent progenitors (Lin⁻cKIT⁺Sca-1⁺CD34⁺CD150⁻CD135⁻CD48⁺) (WT HSPC + WT MSC vs WT HSPC + DBA MSCs: $p=0.098$, $n=4$. DBA HSPC + WT MSC vs DBA HSPC + DBA MSC: $p=0.0225$, $n=4$) (Figure 7D, Supplementary Figure S8C), not long-term HSC (Lin⁻cKIT⁺Sca-1⁺CD34⁻CD150⁺CD135⁻CD48⁺) (Supplementary Figure S8D). However, despite the increase in MPPs, the downstream committed progenitor percentage did not increase (Supplementary Figure S8E). We found upregulated Lin⁻ (Figure 7E, Supplementary Figure S8F), LSK (Figure 7E, F), and MPP (Figure 7F, Supplementary Figure S8G) *in vivo* that is consistent with our *in vitro* results. As expected, LT-HSC was not changed (Supplementary Figure S8G). Unlike *in vitro*, we observed a significantly increased common myeloid progenitor (CMP) population in DBA compared with WT BM, but those progenitors still did not increase GMPs or MEPs (Supplementary Figure S8G). Furthermore, we did not observe an inhibitory effect of DBA MSCs on erythropoiesis (Supplementary Figure S8H), suggesting that DBA MSCs regulate the expansion and differentiation of early HSPCs but do not directly affect erythroid maturation. Taken together, our results indicate that DBA MSCs promote MPP expansion in response to *RPL11* haploinsufficiency, yet MPP commitment is inhibited and does not yield increased downstream progenitors.

Discussion

Despite accumulating evidence of an abnormal BME in IBMFS, the effect of BME on DBA pathogenesis remains poorly understood. In addition, current treatment and research focus on correcting hematologic phenotype, leaving the ongoing effect of defective BME on disease

progression unknown. As gene therapy approaches to cure the disease advance^{44,45}, understanding the long-term effects of an uncorrected bone marrow microenvironment is needed. To fill this gap, we investigated the effect of RPL11 haploinsufficiency in mouse and human MSCs and its association with defects in cell proliferation, differentiation, and HSPC support.

We found p53-independent cell cycle arrest in DBA MSCs, which contrasts with increased p53 reported in DBA erythroids.^{11,12} This may be due to differences in cell types or compensatory mechanisms that overcome p53 stabilization, such as *Mdm2* overexpression found in DEGs from RNA-seq. However, RNA-seq results need to be more thoroughly validated, as we observed differences between gene and protein expression in several targets. This discrepancy may be caused by impaired ribosome biogenesis and a global translation defect observed in DBA. For example, IPA predicted activation of bone differentiation in DBA MSCs based on high *Bmp2* mRNA (Figure 3D). However, BMP2 protein was not elevated (Supplementary Figure S4A), suggesting that these predictions may not be functionally active in DBA MSCs. In contrast, osteopontin was consistently overexpressed across all analyses. While osteopontin alone was sufficient to reduce healthy HSPC expansion, the fold decrease was less than that observed in co-culture (Figure 6A, D). This result implies that other extrinsic factors secreted by DBA MSCs contribute to the simultaneous restriction of HSPC expansion. Nevertheless, osteopontin remains an interesting marker for identifying a defective IBMFS BME. In Fanconi anemia, it was identified as a marker of treatment response,^{46 4646} suggesting a potential role for osteopontin in impaired hematopoiesis in IBMFS.

Lastly, we did not observe a direct association between DBA MSCs and impaired erythroblast maturation (Supplementary Figure S8H). While it is possible that the MSCs do not directly impact erythroblast maturation beyond HSPC commitment to the erythroid lineage, it is also possible that this limitation stems from the lack of additional BM niche cells required for erythroblast maturation.

Maturation of erythroid-committed erythroblasts primarily depends on other niche components not present in our *in vitro* system, such as macrophages and the endothelium^{47,48} in addition to MSCs.

Therefore, our co-culture does not reflect MSC-mediated erythroblast maturation or interactions with

BM niche cells. It is also possible that the erythroblast maturation defect in DBA may be niche-independent and may be intrinsic to RPL11 haploinsufficiency in the erythroid cell itself. With the system we have established in this study, we observed only a direct association between MSCs and uncommitted HSPCs. In this study, we found that DBA MSCs mediate expansion and restrict differentiation at the HSPC level, but not at the erythroid level. However, we propose that DBA MSCs can still reduce overall erythropoietic output by restricting the initial lineage commitment of HSPCs. In conclusion, we propose that RPL11 haploinsufficiency in DBA MSCs is associated with many intrinsic and extrinsic defects. Specifically, DBA MSCs fail to differentiate into osteoblasts and restrict HSPC differentiation through osteopontin and other niche factors. Further follow-up studies are needed to determine whether the abnormal BM milieu can have long-term effects in patients, such as during hematopoietic stem cell transplantation (HSCT) or in the progression of hematologic malignancy. Taken together, our findings provide foundational insights into the molecular defects in DBA MSCs and their association with the disease phenotype.

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Figure 1. Bone marrow-derived mesenchymal stem cells (MSCs) from DBA mice proliferate more slowly due to G2/M phase arrest. (A) Phase-contrast microscopy images of wild-type (WT) and DBA mouse MSCs (DBA). (B) Proliferation curves of Cre-ERT2 (n=5), Mx1-Cre (n=4), and in vitro deleted DBA (+Cre) and WT (-Cre) MSCs (n=3) assessed by Trypan blue exclusion assay. (C) Flow cytometry plots of WT and DBA MSCs stained with CFSE and Ki-67. (D) Representative flow cytometry plots and quantified cell cycle of WT and DBA MSCs (n=3). Quantification of the G2/M phase-regulating genes and protein expression by (E) qPCR (n=4) and (F) Western blot. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA at matched time points. Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001 .

Figure 2. RPL11 haploinsufficient human DBA MSCs proliferate more slowly compared to normal MSCs. Schematic diagrams for RPL11 haploinsufficient human MSC production and microscopic images using (A) CRISPR-Cas9 and (B) patient-derived induced pluripotent stem cells (iPSC). Quantification of RPL11 mRNA by qPCR in (C) CRISPR/Cas9 KO (n=3) and (H) iPSC-derived MSCs (iMSCs) (n=4). Proliferation curves and cell viability of (E) RPL11 KO (n=3) and (F) iMSCs (n=4) assessed by Trypan Blue exclusion assay. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA at matched time points. Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001 .

Figure 3. Bulk RNA sequencing of WT and DBA MSCs. (A) Schematics of the experimental timeline. (B) PCA plots of samples used for the sequencing. (C) Upset plots of differentially expressed genes (DEG) set showing the number of overlapping genes between each time point (0hr, 2 weeks, and 4weeks post-differentiation). Differentially enriched pathways defined using Kyoto Encyclopedia of Genes and Genomes (KEGG) dataset (left) and predicted networks analyzed using Ingenuity Pathway Analysis (IPA, Qiagen) (right) in DBA MSCs compared to WT MSCs at (D) 0hr and (E) 2

weeks of osteoblast differentiation. Differentially expressed genes are filtered with the absolute value of $\log_2(\text{foldchange}) > 1$ and p-adjusted < 0.05 .

Figure 4. Cytokinesis failure is associated with the proliferation defect in Rpl11+/ Δ MSCs. (A)

Confocal microscopic images of mouse WT and DBA MSCs in culture, stained with RPL11 (followed by Alexa Fluor 488 staining for the secondary), Phalloidin-AF647, and DAPI. (B) Quantification of the percentage of binucleated cells in the culture at the time of confocal microscopy. N=4 for WT and n=5 for DBA with more than 50 cells in each image. (C) Time-lapse imaging of WT and DBA MSCs undergoing mitosis using bright-field microscopy. (D) Quantification of cytokinesis failure events observed in the time-lapse imaging. N=4 for WT and n=3 for DBA with at least 4 mitotic events captured in each image. (E) Western blotting of mouse WT and DBA MSCs for MYH9, MYH10 and RhoA. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: * < 0.05 , *** < 0.001 .

Figure 5. YAP inactivation suppresses proliferation and osteoblast differentiation of DBA

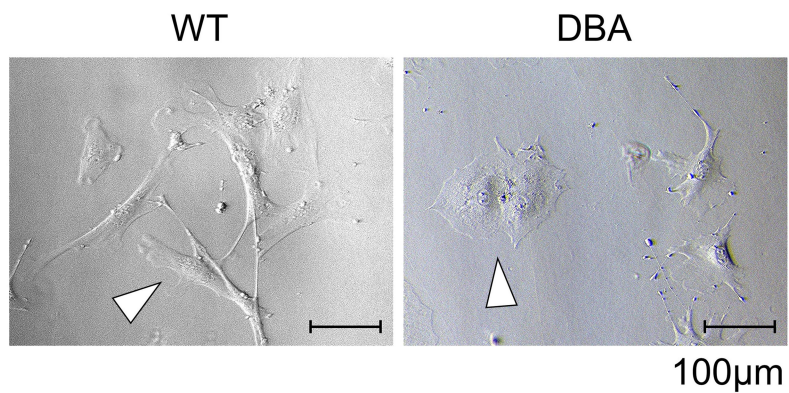
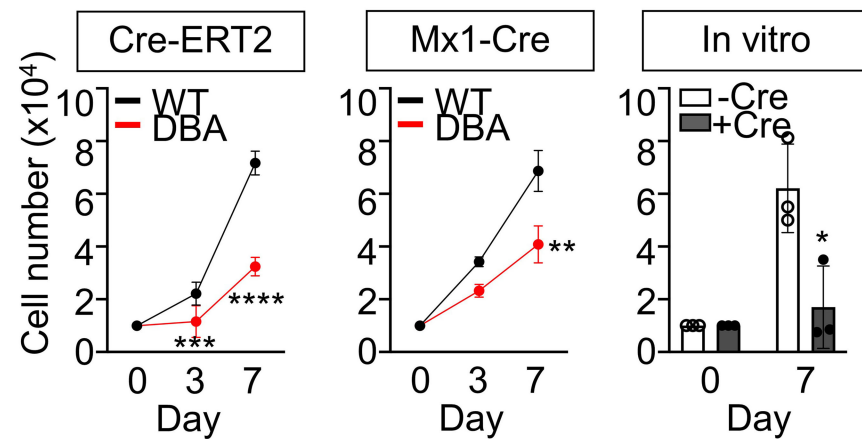
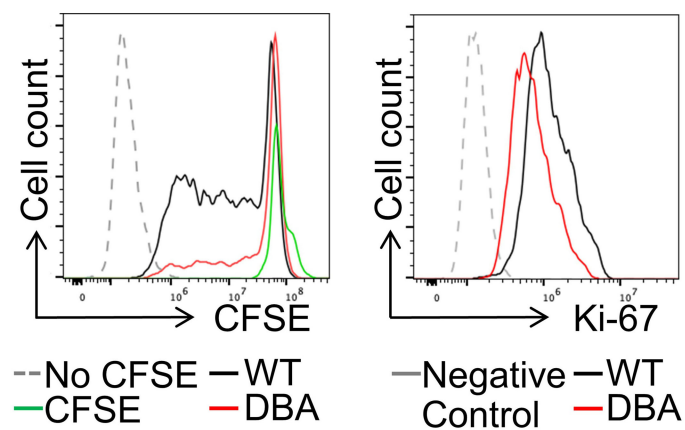
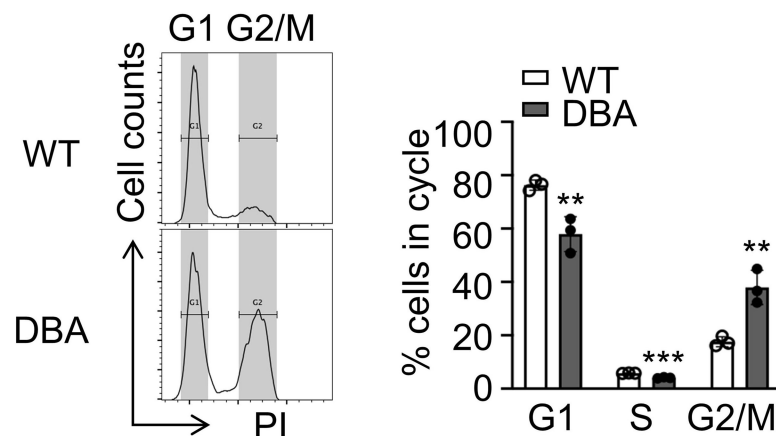
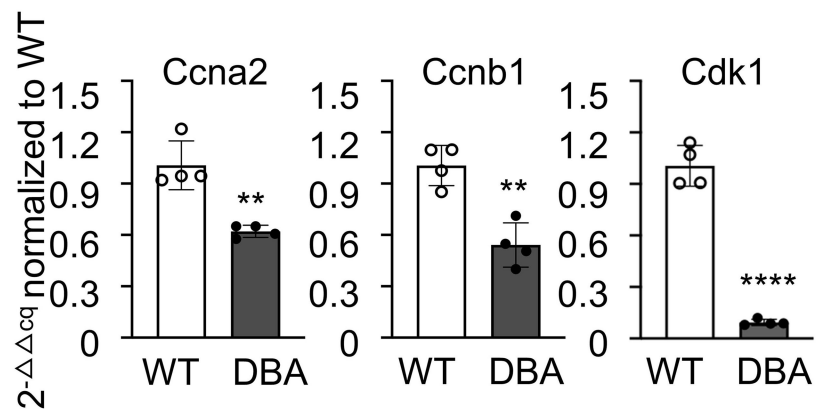
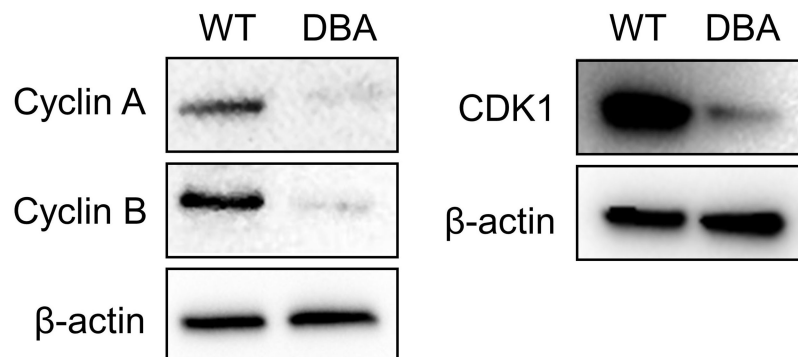
MSCs. (A) Confocal microscopy images of mouse and human control and DBA MSCs stained with an anti-YAP antibody conjugated to Alexa Fluor 488 and DAPI. 20x magnification (B) Phosphorylated and total YAP in mouse WT and DBA cytoplasmic proteins. Quantification of phosphorylated and total YAP is done by densitometric analysis using FlowJo. (C) Microscopic images of human and mouse control and DBA MSCs stained with alizarin red to visualize the calcium deposits produced by mature osteoblasts after 4 weeks of differentiation. (D) Western blotting of WT and DBA with osteoblast differentiation markers RUNX2, osterix, and β -catenin. (E) Proliferation of mouse WT and DBA MSCs treated with TRULI for 7 days was assessed by a trypan blue exclusion assay. (n=6) (F) Brightfield microscopic images of mouse WT and DBA MSCs stained with alizarin red treated with 1 μ M of TRULI for 2 weeks in osteoblast differentiation medium. Scale bars on the images represent 100 μ m. Statistical analysis was performed using a two-way ANOVA with a multiple comparisons test. Error bars represent standard deviation (SD). p-value: **** < 0.0001 .

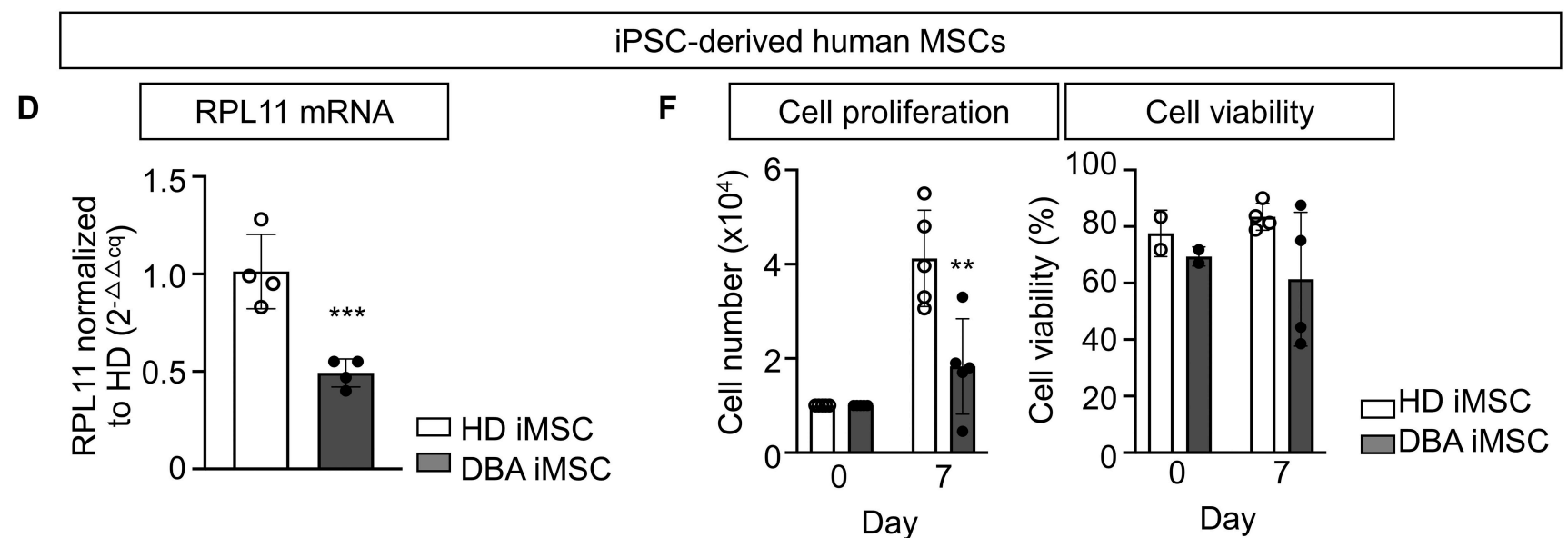
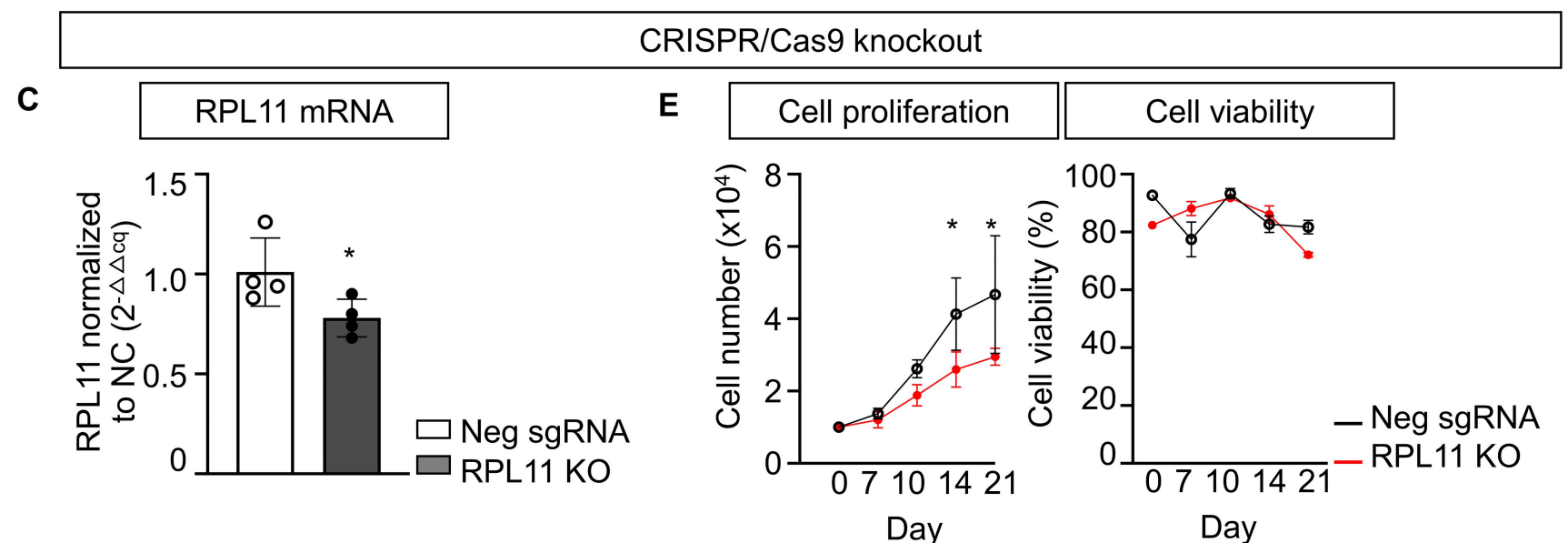
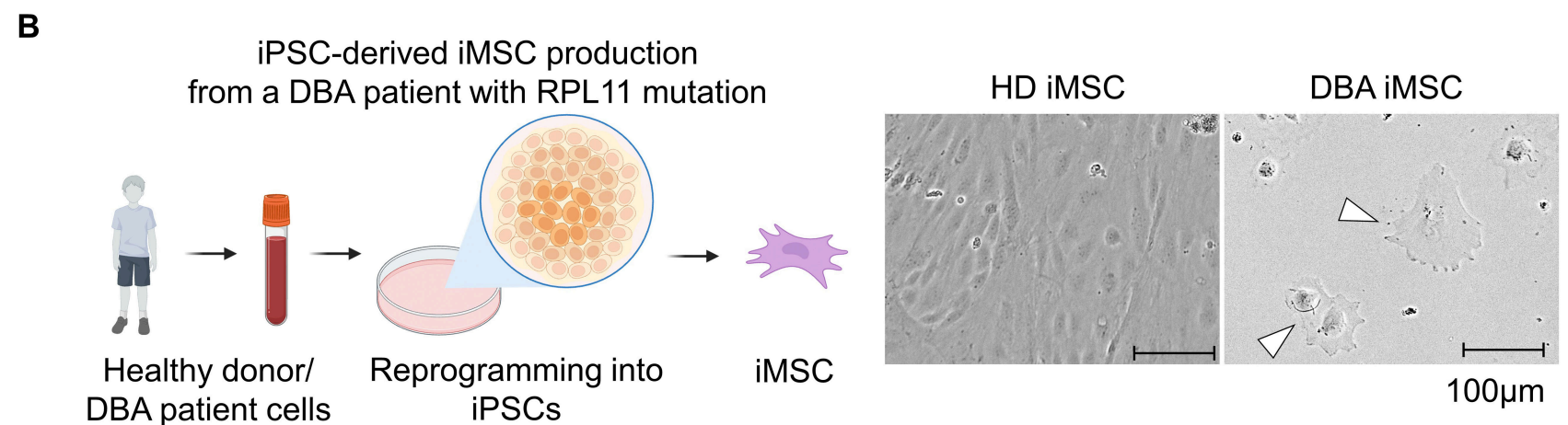
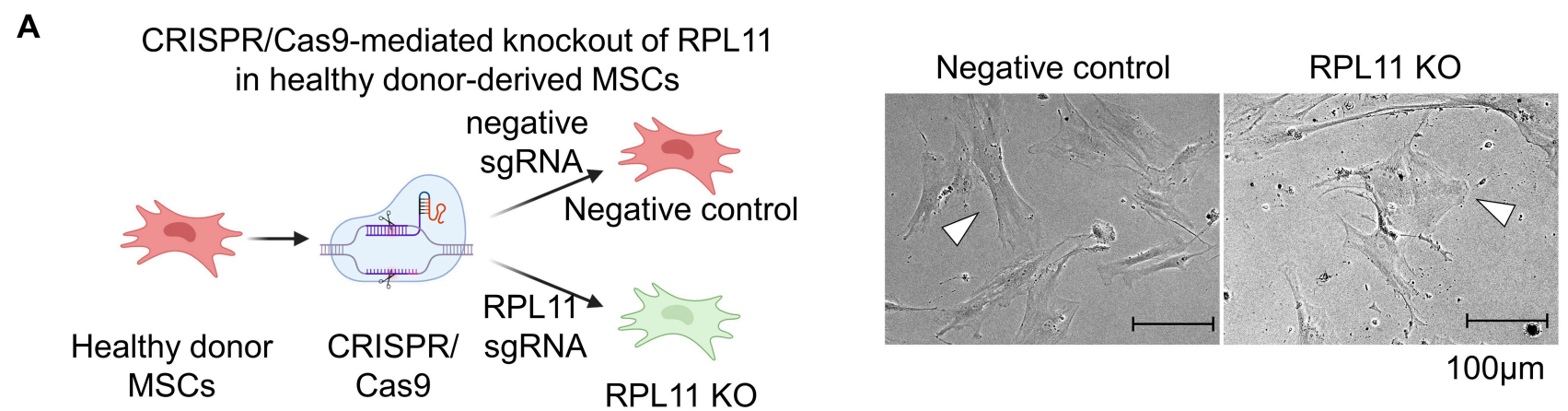
Figure 6. DBA MSCs suppress the expansion of hematopoietic stem and progenitor cells

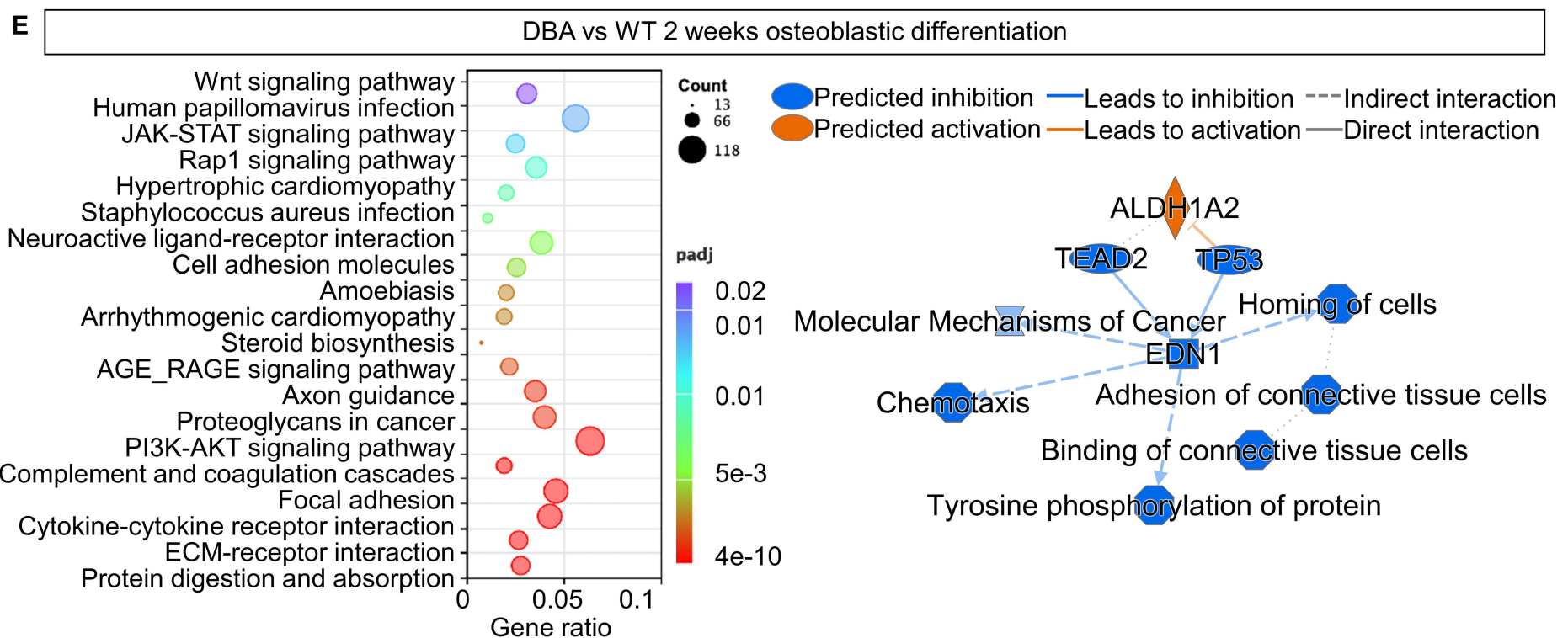
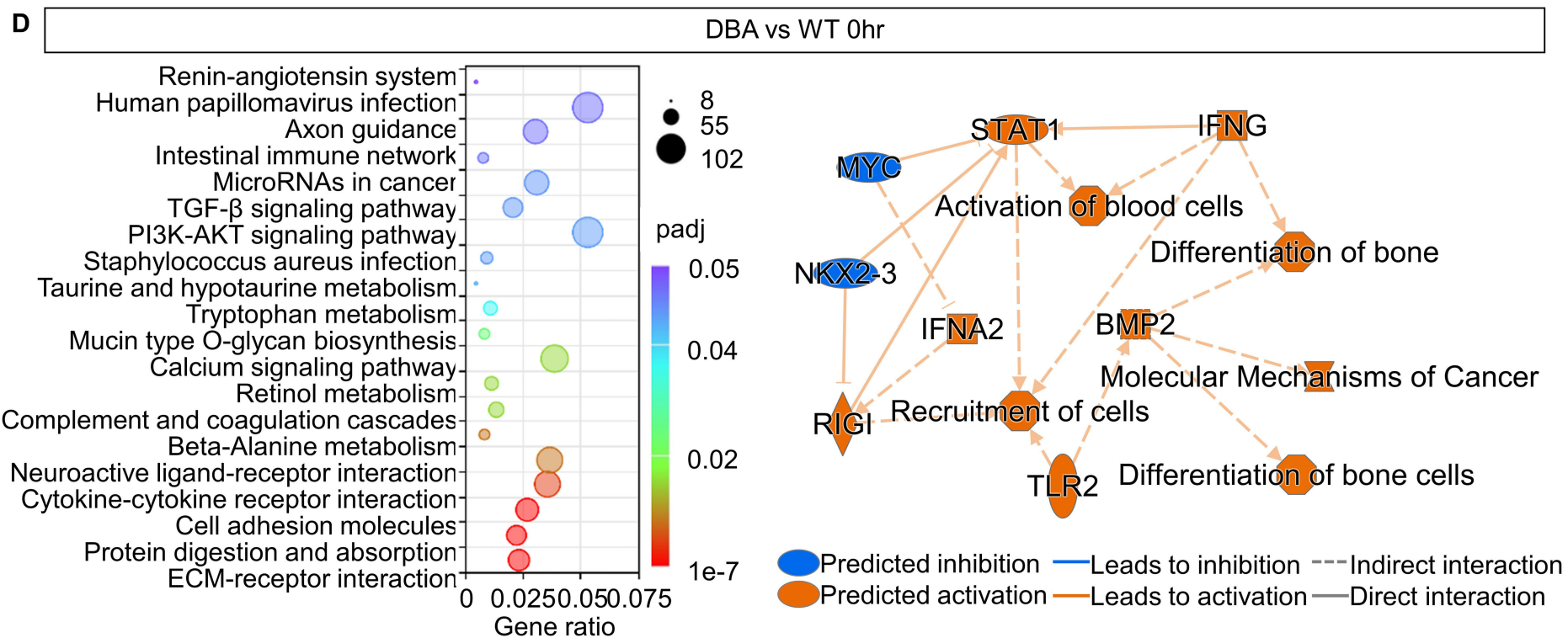
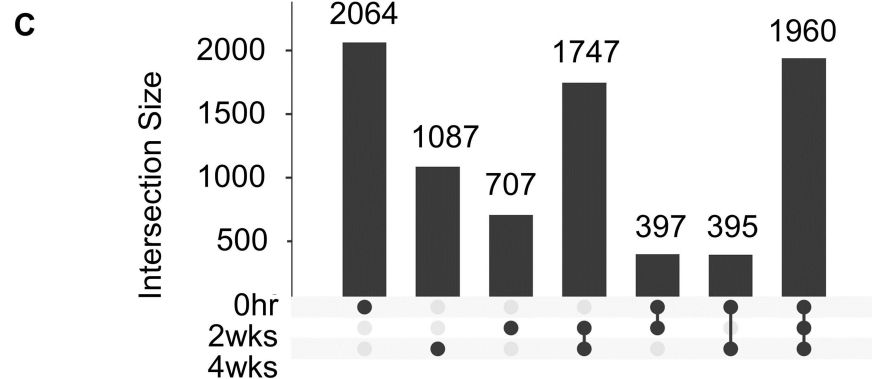
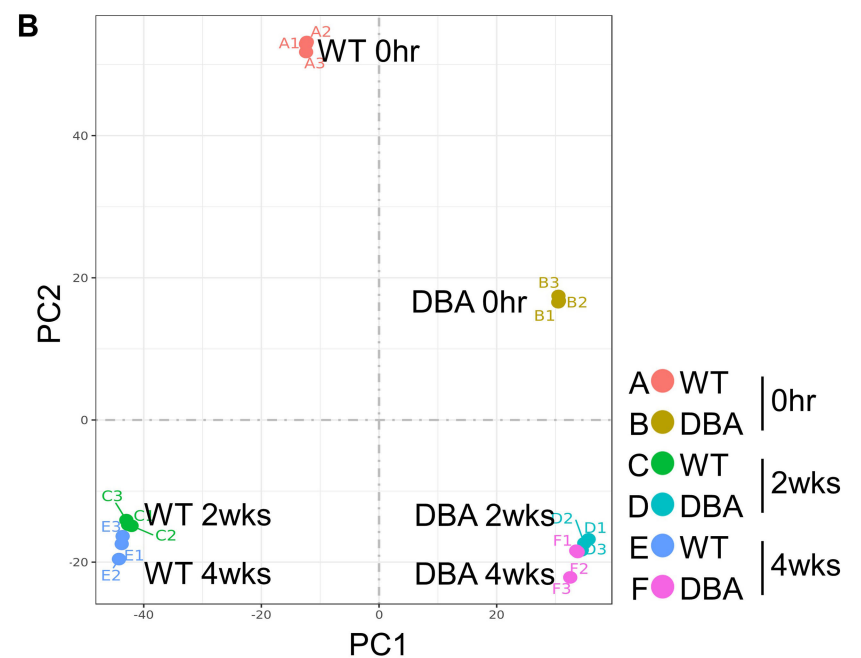
through osteopontin. (A) Cell counts of mouse and human hematopoietic stem and progenitor cells (HSPCs) after 7 days of culture on healthy or DBA MSCs. (B) List of differentially secreted analytes in mouse DBA MSCs compared to control MSCs, and osteopontin protein expression in mouse MSCs. (C) Schematic diagram of osteopontin treatment of mouse and human HSPCs. (D) Cell counts of mouse (n=7) and human HSPCs (n=3) after 7 days of osteopontin treatment. (E) Schematic diagram of anti-osteopontin antibody treatment of HSPCs on MSCs. (F) Cell counts of mouse Lin⁻ cells treated with anti-osteopontin antibody for 7 days on WT or DBA MSCs (n=3). Statistical analysis was performed using Student's t-test (comparing the means between WT and DBA) or a two-way ANOVA with a multiple comparisons test. Error bars represent standard deviation (SD). p-value: *<0.05, **<0.01, ***<0.001, ****<0.0001.

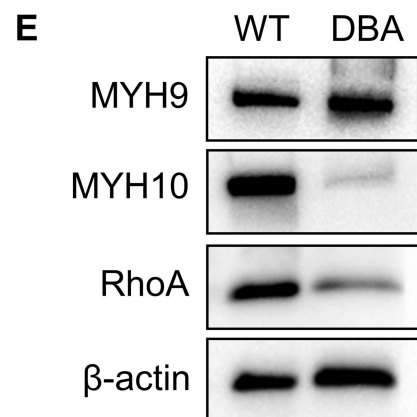
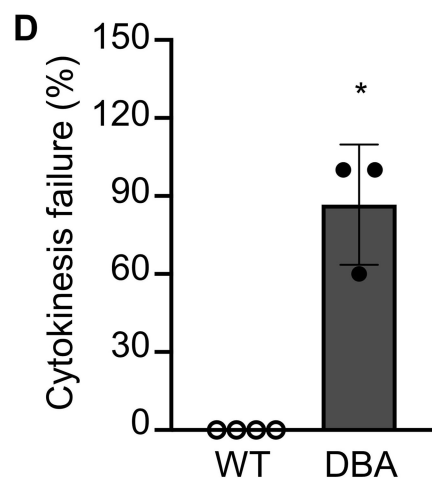
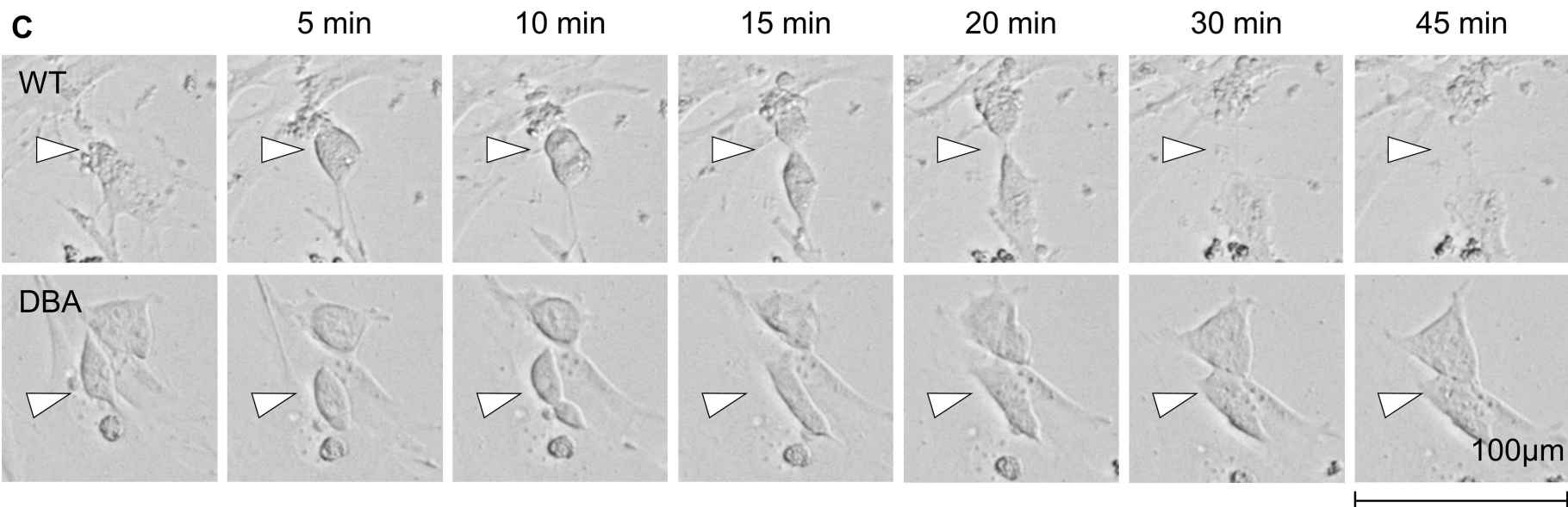
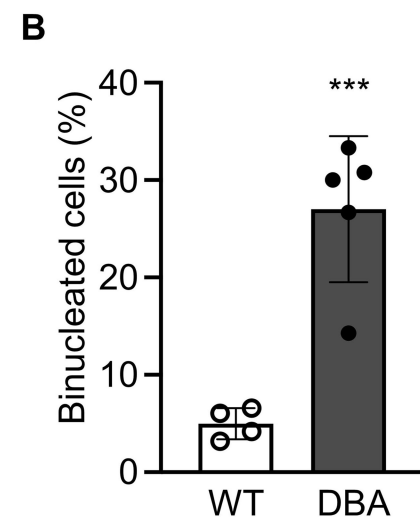
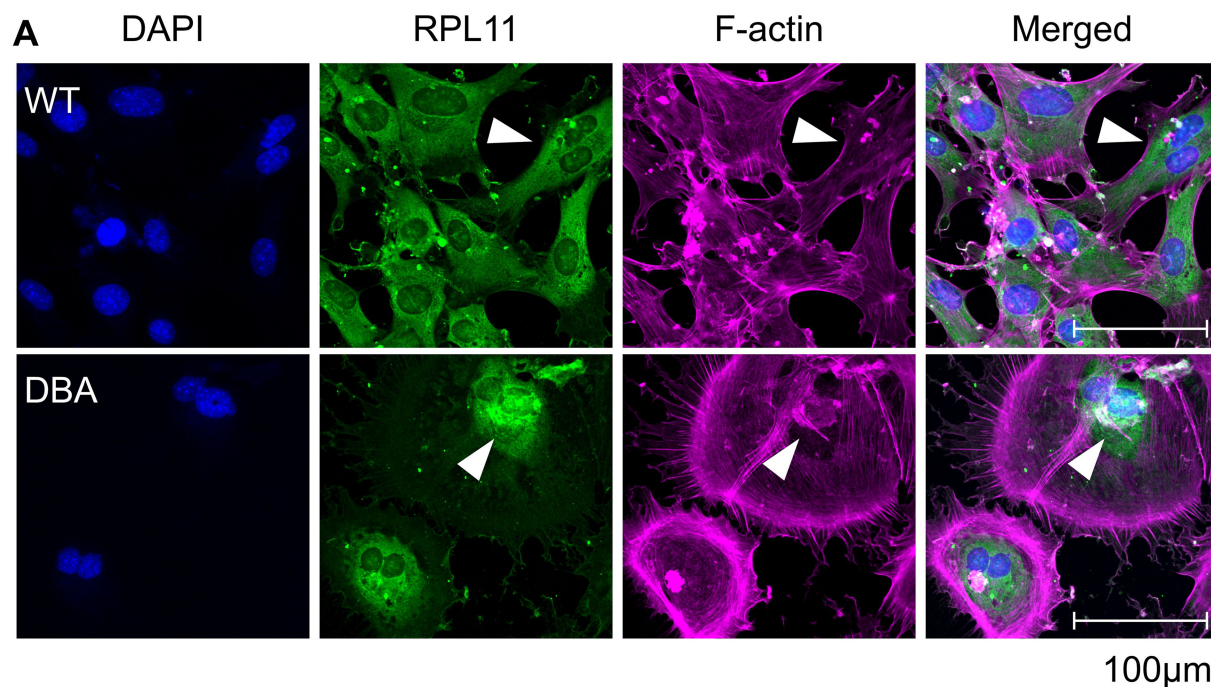
Figure 7. DBA MSCs accumulate multipotent progenitors (MPP) with low differentiation

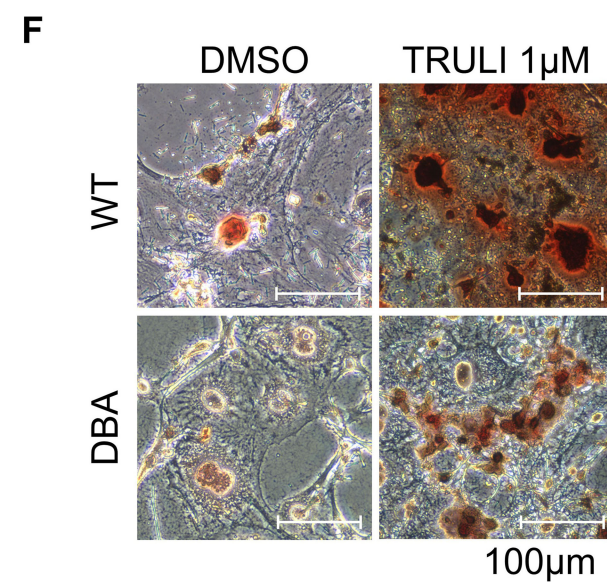
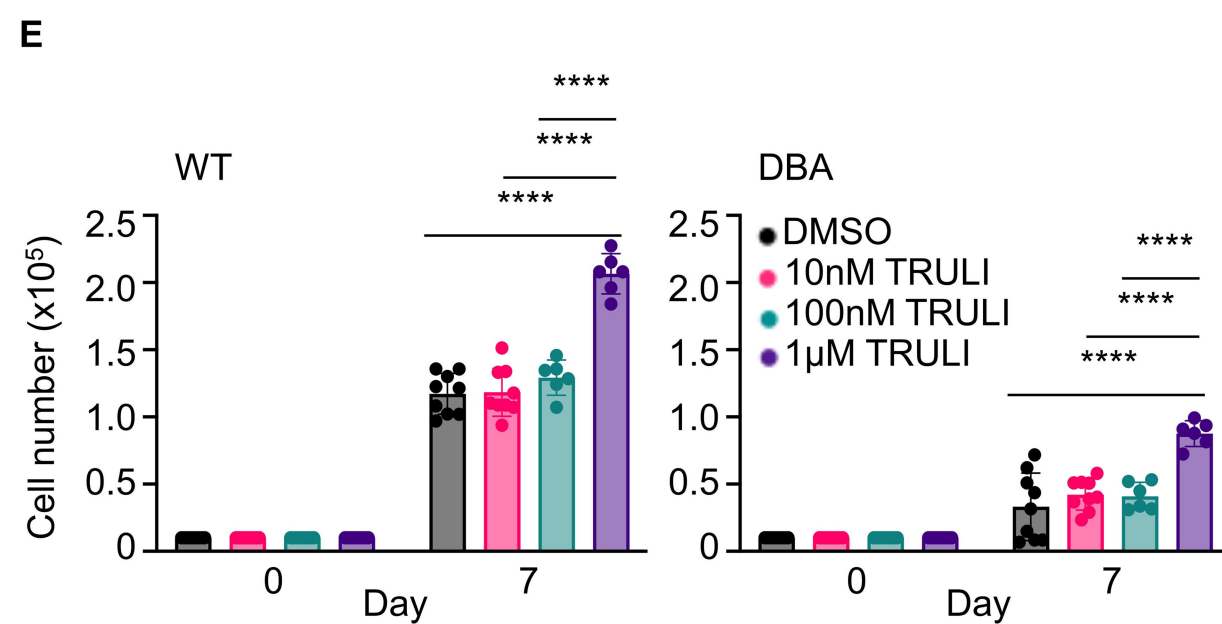
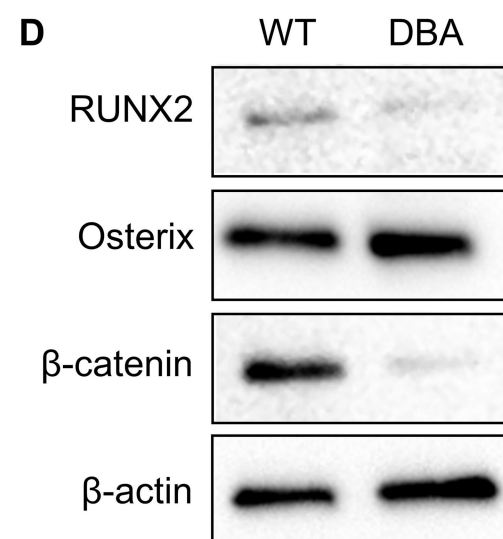
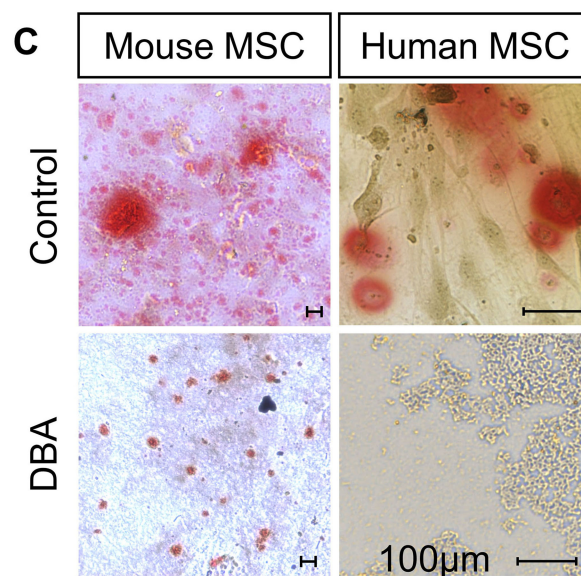
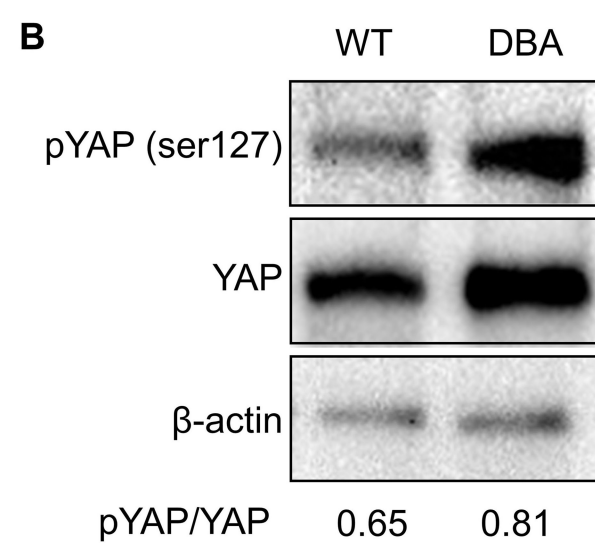
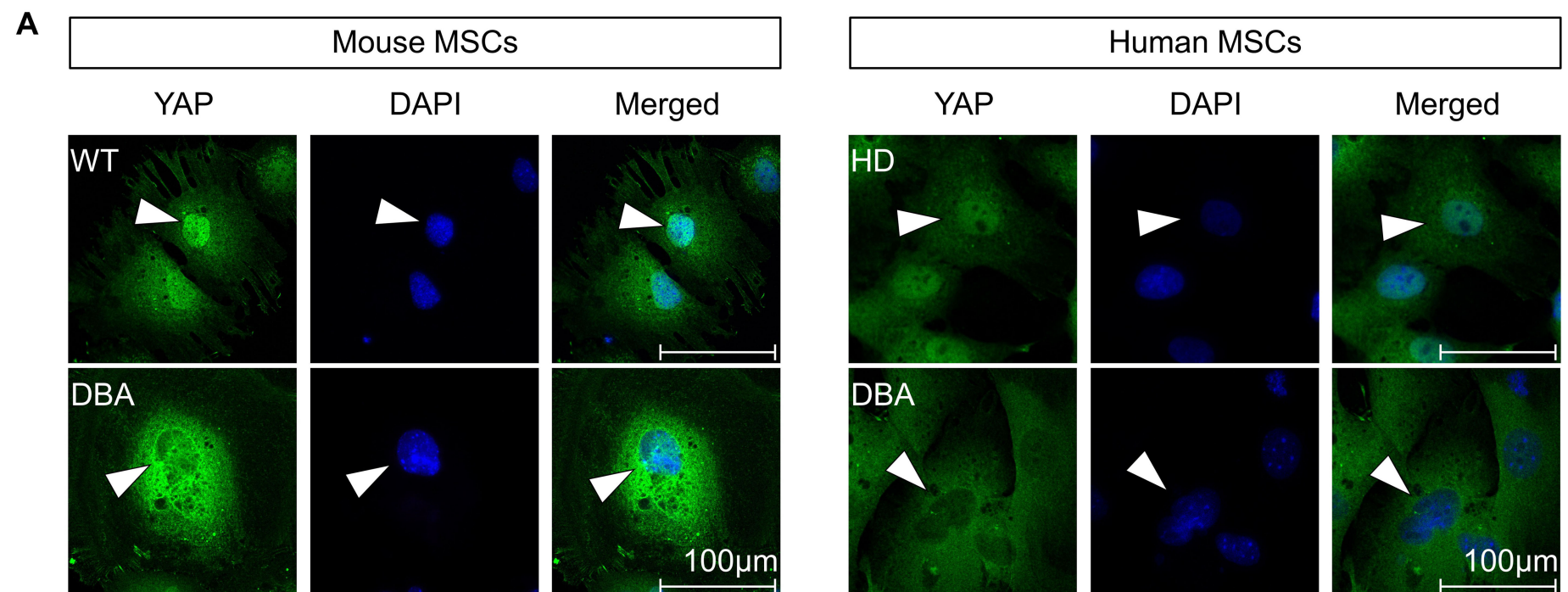
potential. (A) Cell counts of mouse WT and DBA Lin⁻ HSPCs (n=3) cultured on WT or DBA MSCs for 7 days. (B) Percentage of lineage-negative (Lineage⁻) populations within cultured HSPCs assessed by flow cytometry. (C) Representative flow cytometry plots of LSKs within cultured HSPCs using sca-1 and cKIT antibodies. (D) Quantification of LSKs and multipotent progenitors (MPPs) within cultured HSPCs. (E) Representative flow cytometry plots of LSKs within WT and DBA mouse bone marrow (BM). (F) Quantification of LSKs and multipotent progenitors (MPPs) within WT and DBA mouse bone marrow. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: *<0.05, **<0.01, ***<0.001, ****<0.0001.

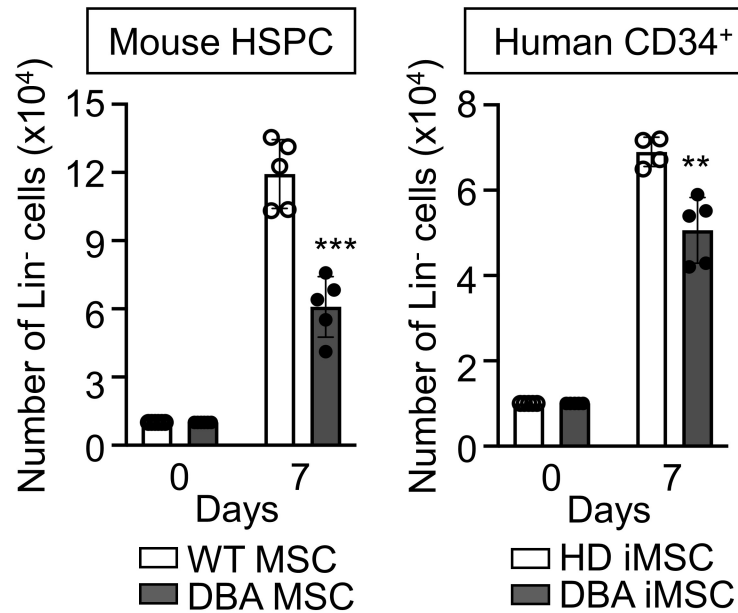
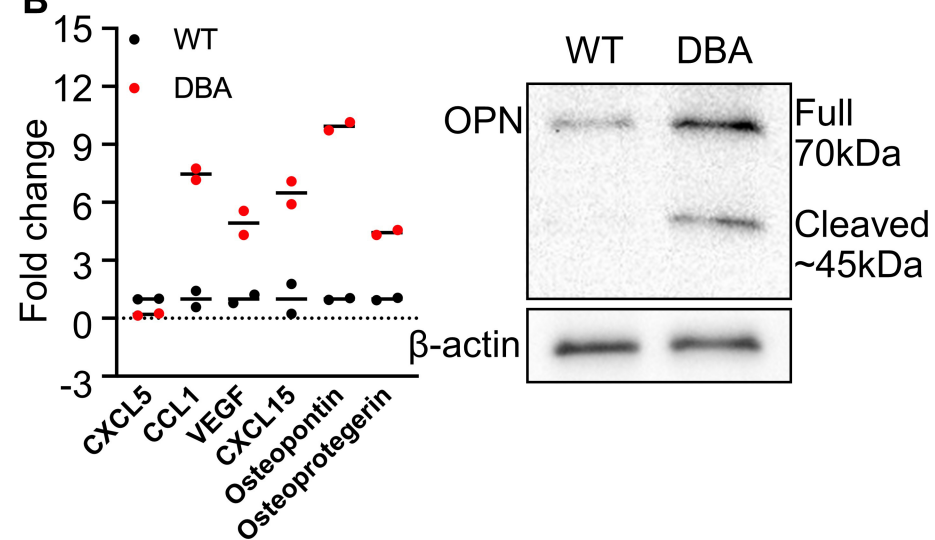
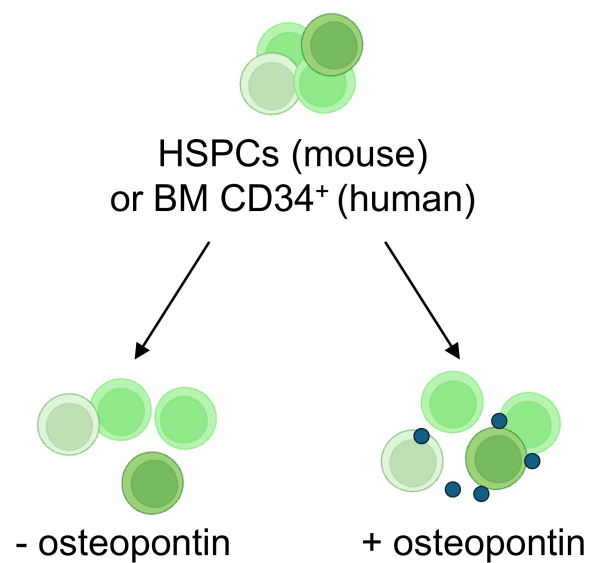
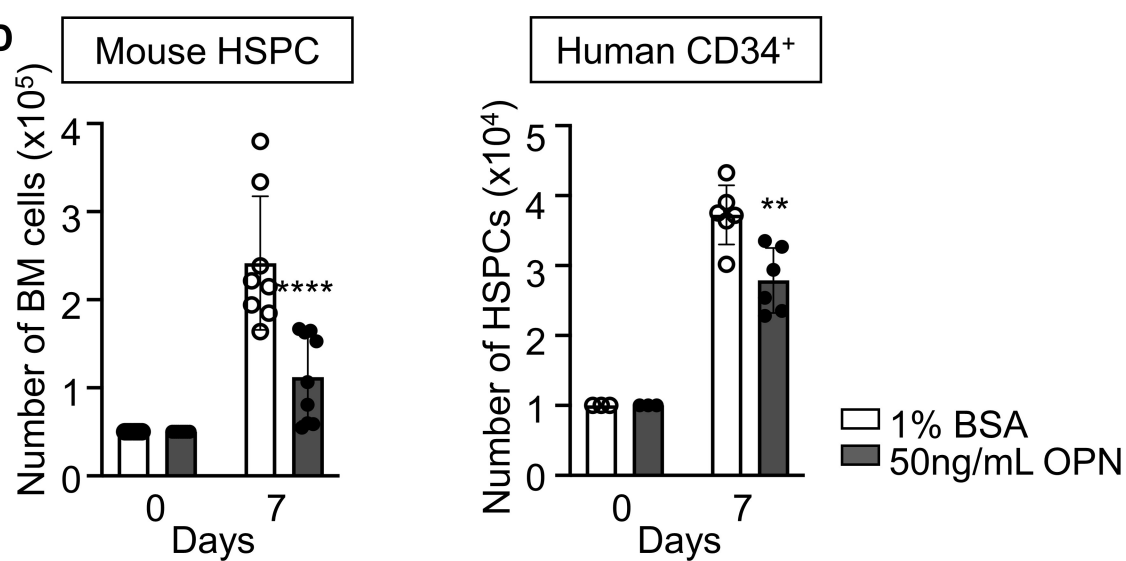
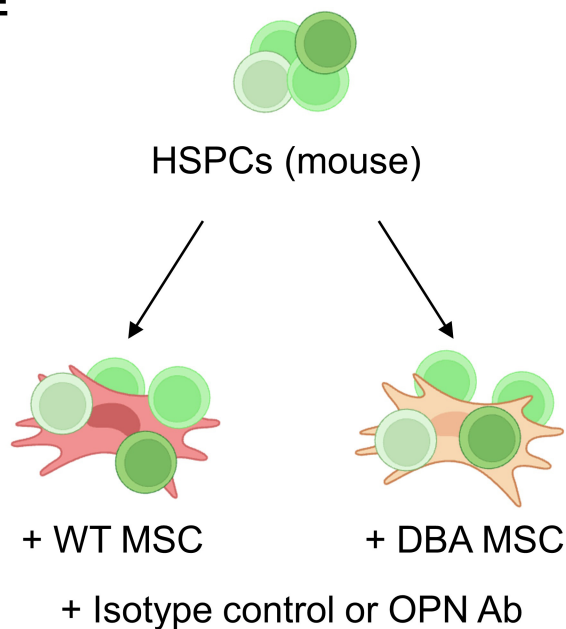
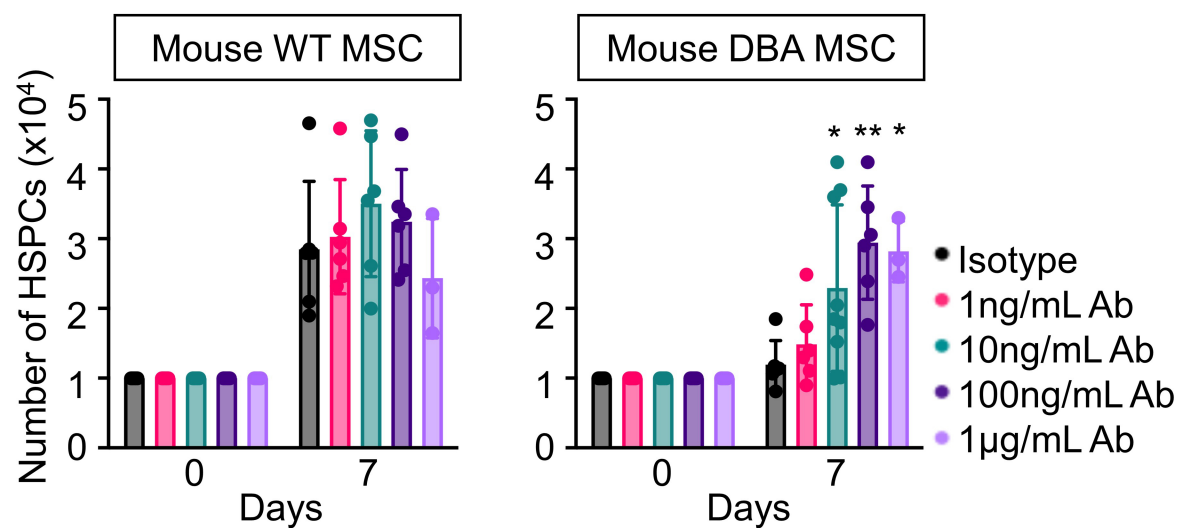
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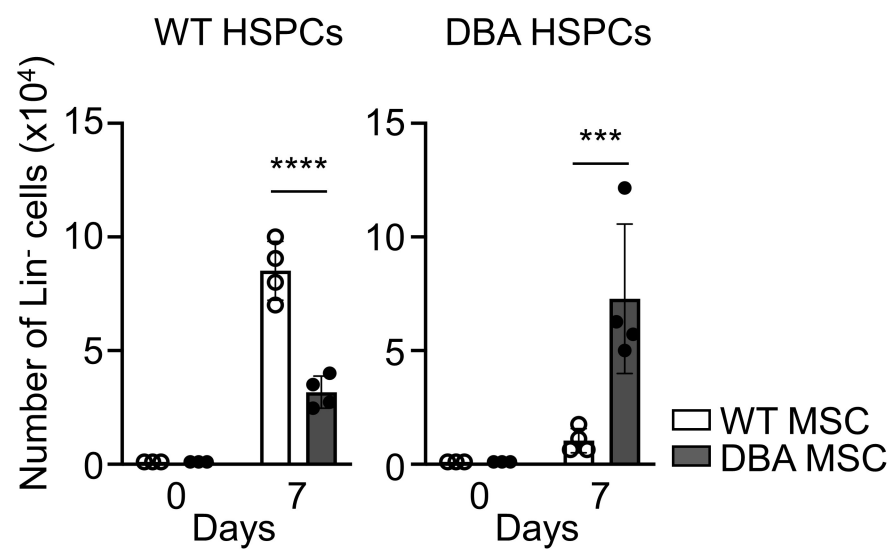
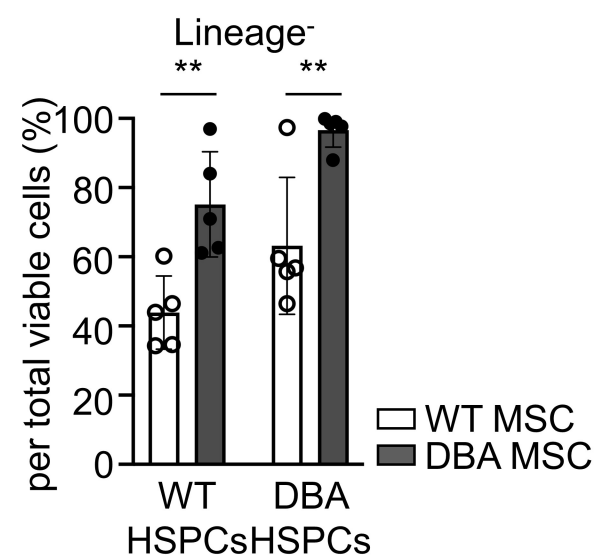
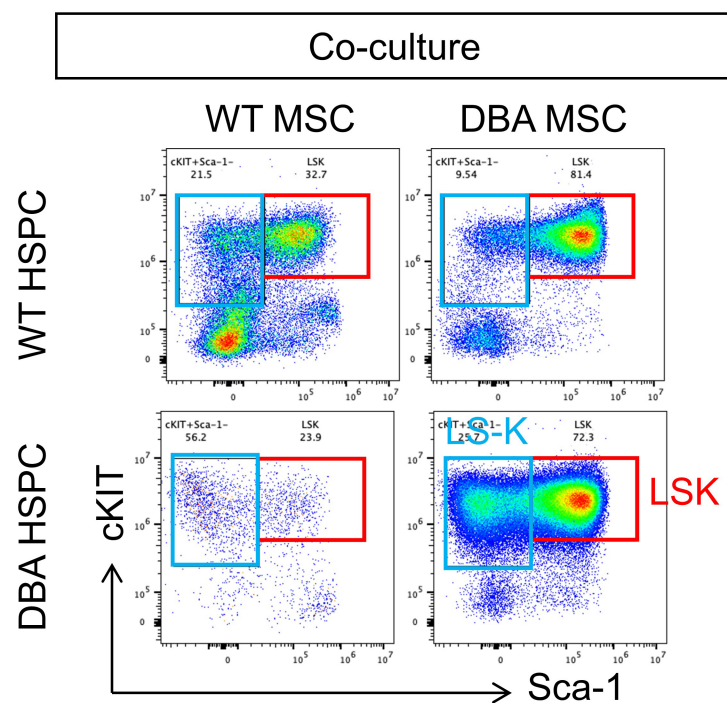
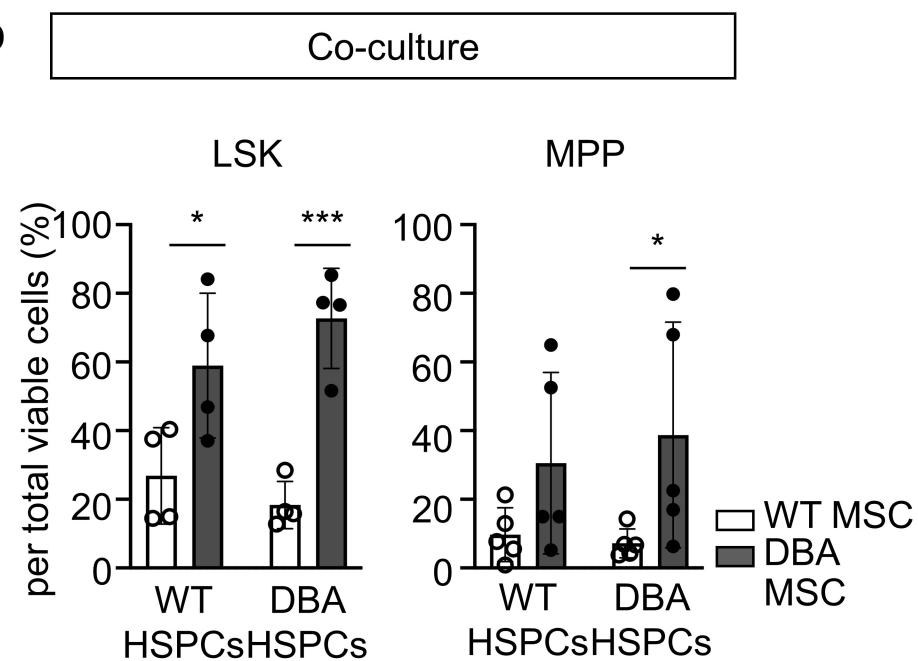
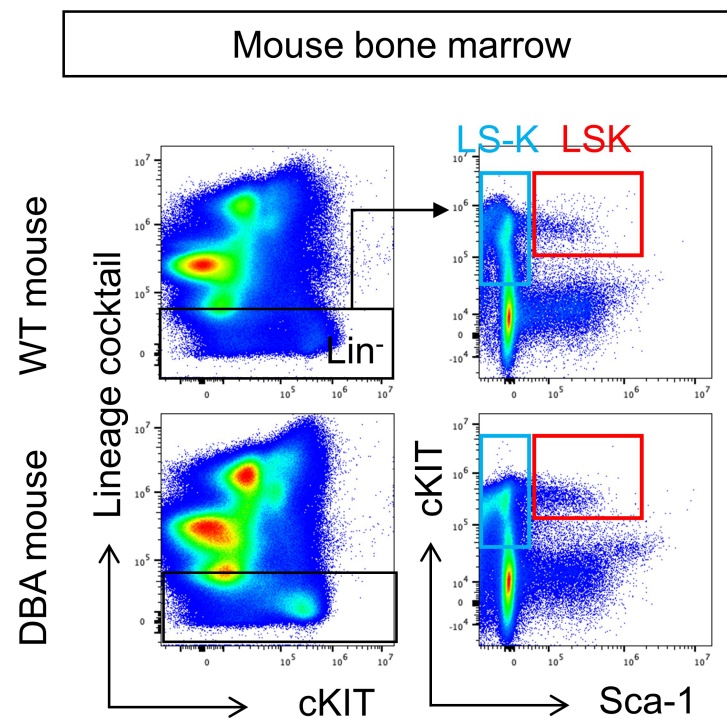
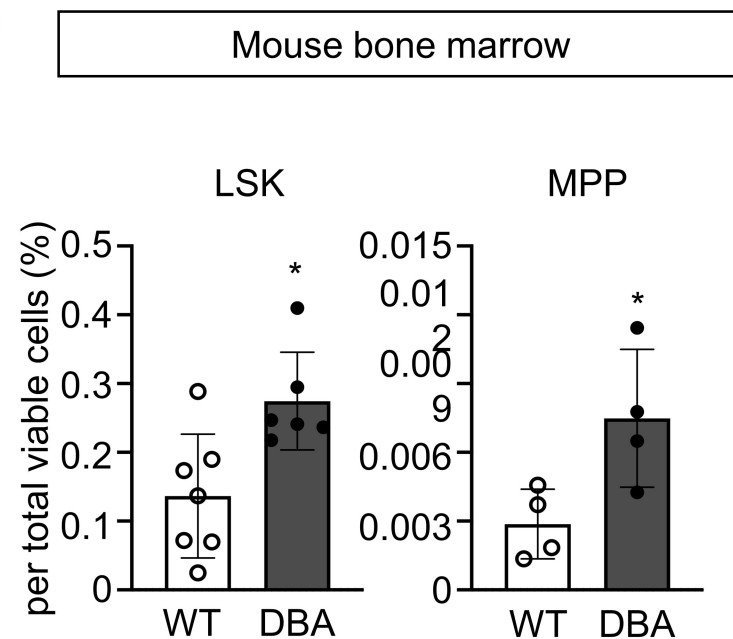








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Supplemental Methods

Transgenic mouse models for Rpl11 haploinsufficient Diamond-Blackfan anemia.

Mice harboring loxP sites for *Rpl11* exons 3 and 4 were crossed with Cre-ERT2 or Mx1-Cre mice. Recombination was induced in 8-day-old *Rpl11*-Cre-ERT2 or Mx1-Cre mice with Tamoxifen or polyinosinic: polycytidylic acid (poly I:C), respectively (Supplementary Figure 1A). Red blood cell (RBC) and hemoglobin (Hb) were monitored by complete blood count (CBC) until mice presented anemia (Hb < 10g/dL in Cre-ERT2, <8g/dL in Mx1-Cre) (Supplementary 1B-E).

Human induced pluripotent stem cells (iPSC) generation.

RPL11 patient iPSCs were generated as previously described²⁶. In brief, 5×10^5 of peripheral blood mononuclear cells (PBMCs) were added to a 24-well plate in 1 ml of PBMC media (RPMI, 10% FBS, 10 ng/ml of GM-CSF, IL-3, IL-6, FLT3L, TPO, and SCF). After 7 days, 1×10^5 of PBMCs were replated in 500 μ l of medium and inoculated with Sendai virus²⁶ (a gift from Dr. Mahito Nakanishi, at the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) encoding KLF4, OCT4, SOX2, and C-MYC at a multiplicity of infection (MOI) = 1. After 48 hours, cells were replated into a 6-well plate containing 1:1 of PBMC:hiPSC media (DMEM/F12, 20% KnockOut Serum Replacement, 1% non-essential amino acids, 1% P/S, 100 μ M 2-mercaptoethanol, and 5 ng/mL FGF2). Media were changed daily for 16 days. Then, dissociated colonies were transferred to mouse embryonic fibroblast (MEF)-coated plates prepared the day before (1.5×10^4 cells/cm²) in hiPSC medium, with 10 μ M Y-27632 (ROCK inhibitor) for the first 24 hours. After 5 days, cells were dissociated using TrypLE and transferred to iMatrix-511-coated plates in StemFit medium (AmsBio, UK) supplemented with 10 μ M Y-27632 for 24 hours. Thereafter, hiPSCs were passaged every 4–6 days at 5×10^3 to 1×10^4 cells/cm².

Generation of iMSCs from iPSCs

Healthy donor and DBA patient iPSC colonies were transferred to a tissue culture plate (6-well) in MSC medium (1:1 of MesenCult: low glucose DMEM, 10% FBS, L-Glutamine, penicillin/streptomycin). The growth medium was replaced every other day, and morphological changes were observed daily. After 7 days, or once the cells reached confluence, the cells were trypsinized for characterization. Cells validated for surface markers and differentiation potential are defined as iMSCs and used in the study.

Real-time quantitative polymerase chain reaction (qPCR)

RNA was extracted from MSCs using the RNeasy mini (Qiagen) and transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The quantitative PCR reaction was performed with SYBR Green Master Mix (Bio-Rad) on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). 7SL was used as an internal control for mouse and human mRNA. Fold change in mRNA was calculated by the comparative C_q method and normalized to normal controls.

Supplemental Methods

Western blot analysis

15 µg of protein extracted using RIPA buffer (Thermo Fisher) was loaded onto a 4-15% polyacrylamide gel (Bio-Rad) and run at 110V for 30 to 40 minutes, then transferred onto PVDF membranes using a semi-dry transfer cell (Bio-Rad) at 10V for 30 minutes. Membranes were blocked in 5% milk for 1 hour, incubated with the primary antibody diluted in 5% milk for 1.5 hours, washed with TBST, and incubated with secondary antibody for 1.5 hours, all at room temperature. Membranes were washed with TBST and visualized with chemiluminescent substrates (SuperSignal West Pico PLUS, Thermo Fisher) using ChemiDoc MP imaging system (Bio-Rad). If needed, membranes were stripped (Restore™ Buffer, Thermo Fisher) for 15 minutes and re-probed.

Brightfield, phase-contrast, time-lapse imaging, and confocal microscopy

Brightfield, phase-contrast, and time-lapse images were acquired using a Keyence BZ-X microscope at 2x or 10x magnification in their experimental plates. For time-lapse imaging, 1×10^4 of mice MSCs were seeded onto a 12-well tissue culture plate. After 3 days of culture, cells were washed with DPBS, and 0.5mL of MSC medium was added. Z-stack images were acquired every 5 minutes for 3 hours at 37°C using the incubation chamber and the microscope's live-imaging function.

For confocal microscopy, 1×10^4 of MSCs were seeded in an 8-chamber slide. After 24 hours, cells were fixed with 4% paraformaldehyde for 30 minutes, followed by washing, 1-hour blocking (1X PBS, 5% BSA, and 0.3% Triton X-100), and primary antibody staining (overnight at 4°C). The next day, cells were washed twice with PBS and incubated with secondary antibody for 1 hour. Stained cells were washed, stained with 5 µg/mL DAPI for 10 minutes, and mounted with #1.5 cover glass using hardening mounting medium (Prolong Gold, Thermo). Stained slides were imaged using a Leica Stellaris 8 DIVE upright confocal microscope. Image analysis (counting) or resizing was done by FIJI. A list of antibodies is provided in Supplementary Table 1.

RNA-sequencing and analysis

mRNA was isolated from MSCs differentiated for 0 hours, 2 weeks, and 4 weeks using the RNeasy Mini kit (Qiagen). Subsequent sequencing and bioinformatic analyses were carried out at Novogene. In brief, the library was prepared using poly-T oligo-attached magnetic beads. After fragmentation, cDNA was synthesized using random hexamer primers, followed by the second-strand cDNA synthesis using dTTP. Quantified libraries were sequenced on the Illumina NovaSeq X Plus platform (PE150) and CASAVA (v1.8). QC-filtered reads (mean=53M) were aligned to the reference genome (mm39) using HISAT2. Gene expression was estimated by Fragments Per Kilobase of transcript sequence per Million mapped fragments (FPKM) method. Differential gene expression (DEG) was analyzed using DESeq2, with absolute $\log_2(\text{FoldChange}) > 1$ and adjusted p-value < 0.05 . KEGG analysis was done using clusterProfiler (v4.8.1) with adjusted p-value < 0.05 . Ingenuity Pathway Analysis (Qiagen) was performed using DEGs. UpSet plots were used to show intersections of DEGs between 0 and 4 weeks post-differentiation, using the R package UpSetR (v.1.X.X). RNA-sequencing data are available at GEO under accession number GSE325350.

Antibody-based secretome array and ELISA

1×10^4 of MSCs were seeded onto a 24-well tissue culture plate. After 48 hours, the supernatant was collected and spun at 1200rpm for 5 minutes. The collected supernatant was used for the Mouse Cytokine Antibody Array (RayBio, AAM-CYT-1000) or ELISA (Invitrogen, ERA46RB) following the manufacturer's instructions.

Supplemental Table 1. Primary and secondary antibodies used for confocal microscopy

Target	Vendor	Dilutions	Catalog #
RPL11	Proteintech	1:50	16277-1-AP
Phalloidin eFluor 660	eBioscience	1x	50-6559-05
DAPI	Biolegend	5ug/mL	422801
YAP	Thermo Fisher	1:100	MA5-35282
α-tubulin	Proteintech	1:100	11224-1-AP
Lamin A/C	Proteintech	1:50	CL488-10298
Alexa Fluor 488	Invitrogen	1:500	A11008

Supplemental Table 2. Antibodies used for flow cytometry

Target	Fluorochrome	Isotype	Vendor	Catalog #
CFSE			Invitrogen	C34554
Ki-67	Alexa Fluor 488	Rat IgG2b, κ	BioLegend	151204
Propidium iodide			BioLegend	421301
Annexin V	PE		BioLegend	640907
7 AAD			BioLegend	420403
cKIT	PE/Cy7	Rat IgG2b, κ	BioLegend	105814
CD34	PerCP/Cy5.5	Armenian Hamster IgG	BioLegend	128607
Sca-1 (Ly-6A/E)	Brilliant Violet 711	Rat IgG2a, κ	BioLegend	108131
Lineage Cocktail	Pacific Blue	Rat IgG2a/IgG2b	BioLegend	133306
CD16/32	APC/Cy7	Rat IgG2a, λ	BioLegend	101328
CD48	APC	Armenian Hamster IgG	BioLegend	103412
CD135	PE/Cy5	Rat IgG2a, κ	BioLegend	135312
CD150	PE	Rat IgG2a, λ	BioLegend	162606
CD44	FITC	Rat IgG2a, κ	BioLegend	156008
CD71	PE	Rat IgG2a, κ	BioLegend	113808
Ter119	APC/Cy7	Rat IgG2b, κ	BioLegend	116223
CD45	PE/Cy7	Mouse IgG1, κ	BioLegend	157613
CD146	PerCP/Cy5.5	Rat IgG2a, κ	BioLegend	134710
CD29	Pacific Blue	Armenian Hamster IgG	BioLegend	102224
CD90	APC	Rat IgG2a, κ	BioLegend	166405
CD105	PE/Dazzle	Rat IgG2a, κ	BioLegend	120424
CD146	PE/Cy7	Mouse IgG1, κ	BioLegend	361007
CD29	PerCP/Cy5.5	Mouse IgG1, κ	BioLegend	303024
CD45	FITC	Mouse IgG1, κ	BioLegend	304006

Supplemental Table 2. Antibodies used for flow cytometry (Continued)

Target	Fluorochrome	Isotype	Vendor	Catalog #
CD73	BV711	Mouse IgG1, κ	BioLegend	344026
CD90	APC	Mouse IgG1, κ	BioLegend	389805
CD105	PE/Dazzle	Mouse IgG1, κ	BioLegend	323224

Supplemental Table 3. Antibodies used for Western blot

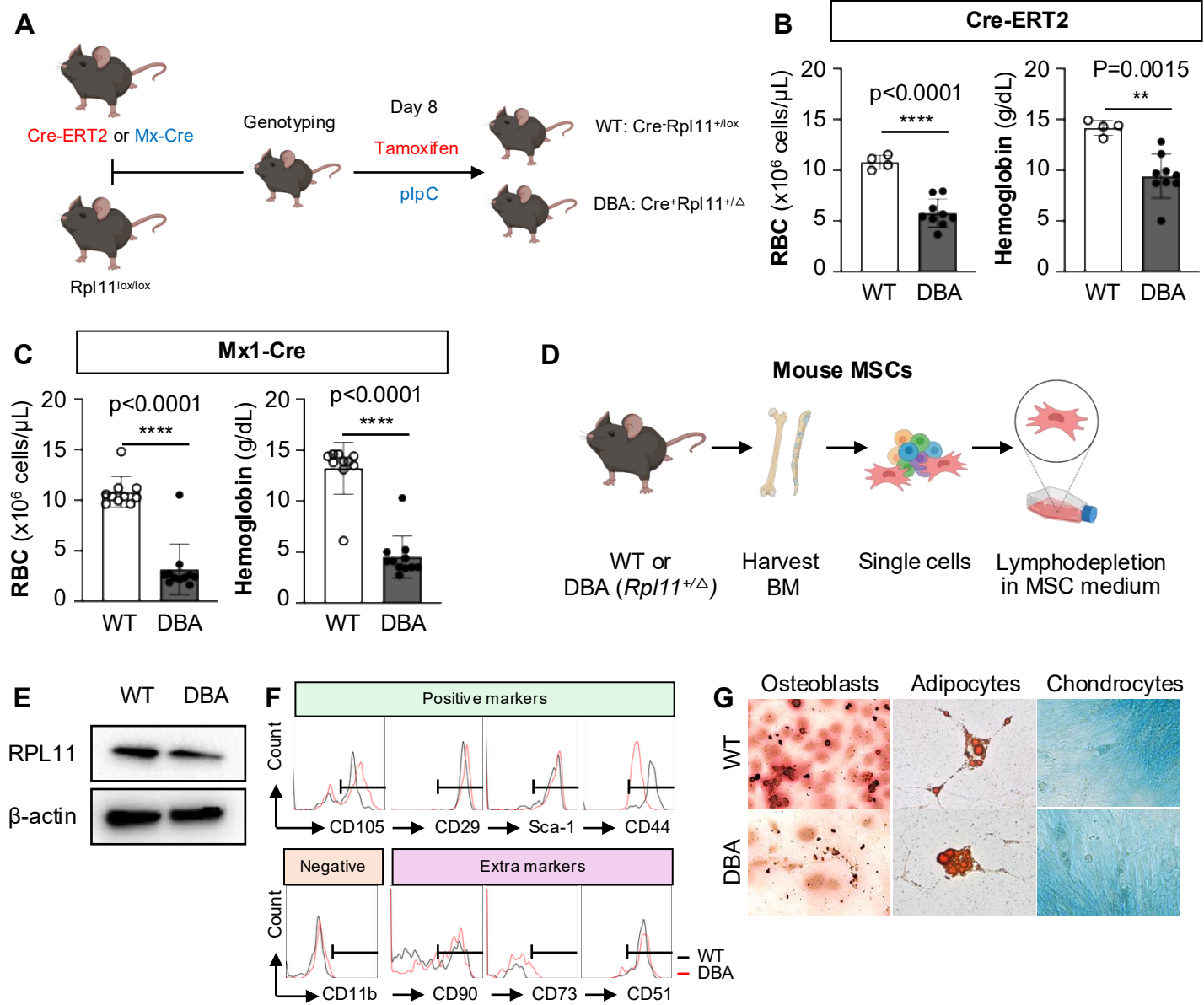
Target	Vendor	Dilutions	Catalog #
Cyclin A2	Proteintech	1:5000	18202-1-AP
Cyclin B1	Proteintech	1:1000	55004-1-AP
CDK1	Cell Signaling	1:1000	77055
CDK2	Proteintech	1:500	60312-1-Ig
Cyclin E1	Cell Signaling	1:1000	20808
Cyclin D1	Cell Signaling	1:1000	55506
p53	Cell Signaling	1:1000	2524
p21	Cell Signaling	1:1000	37543
β-actin	Proteintech	1:20000	66009-1-Ig1
MYH9	Proteintech	1:2000	60233-1-Ig
MYH10	Proteintech	1:1000	67243-1-Ig
RhoA	Proteintech	1:1000	10749-1-AP
Phospho-YAP (ser127)	Cell Signaling	1:1000	13008
YAP	Thermo Fisher	1:10000	MA5-35282
RUNX2	Proteintech	1:2000	82636-2-RR
Osterix (Sp7)	Proteintech	1:5000	85934-1-RR
β-catenin	Cell Signaling	1:1000	8480
Osteopontin	Proteintech	1:1000	83341-1-RR

Supplemental Table 3. Antibodies used for Western blot (Continued)

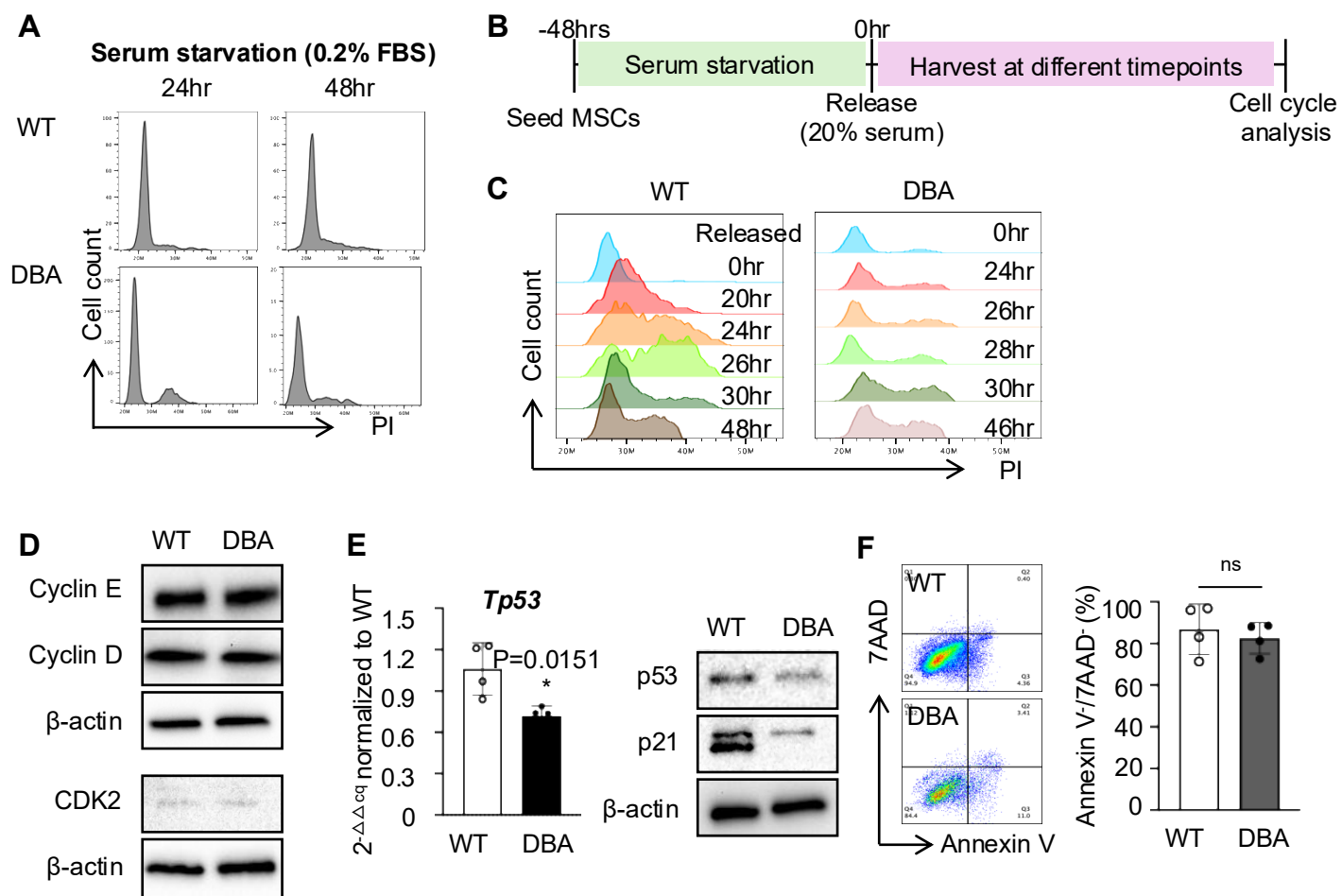
Target	Vendor	Dilutions	Catalog #
BMP2	Selleckchem	1:1000	F2206
BMP4	Proteintech	1:200	12492-1-AP
BMP7	Thermo Fisher	1:1000	MA5-52761

Supplemental Table 4. Other reagents and drugs used for the experiment

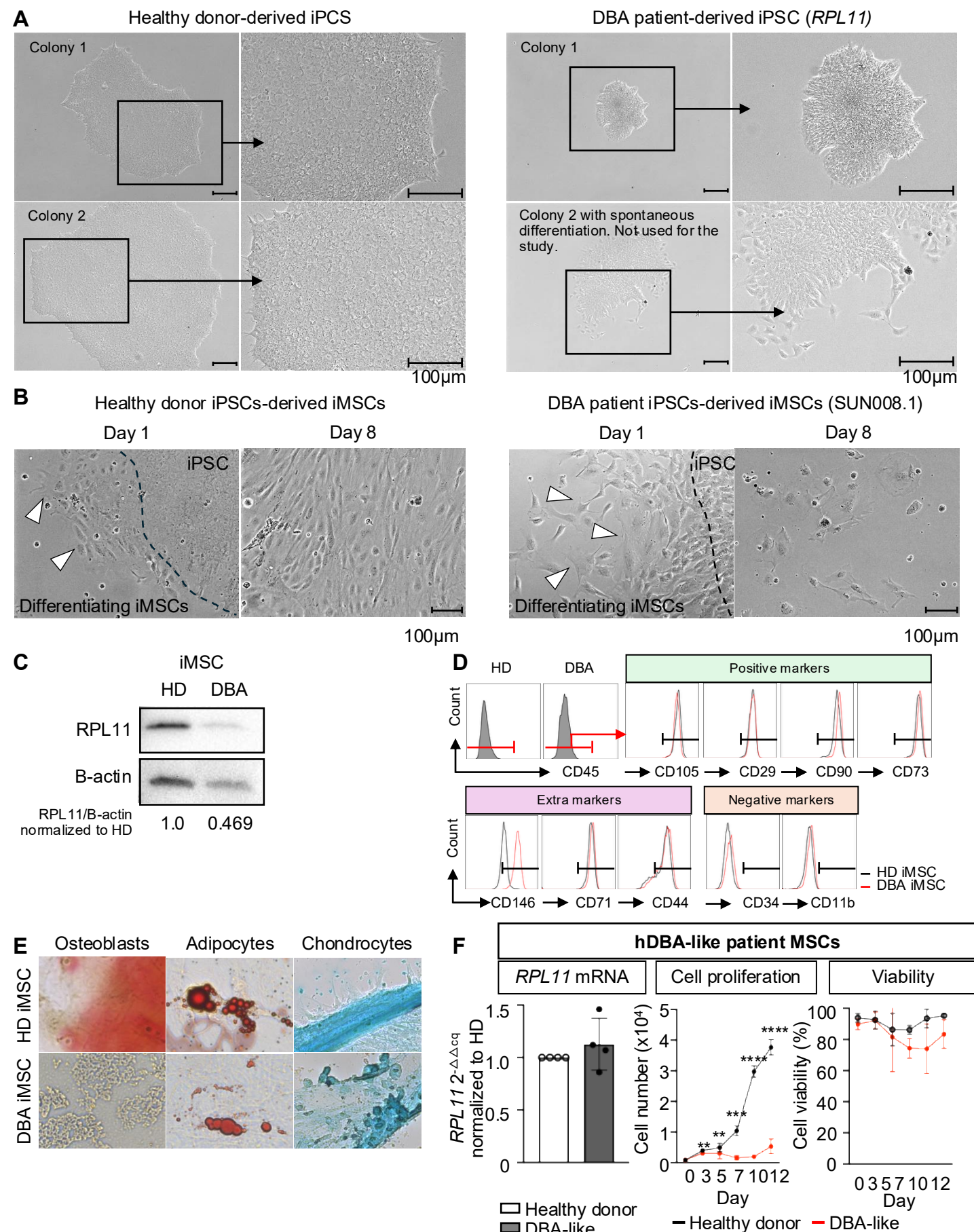
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TRULI	MedChem Express	HY-138489
Recombinant human Osteopontin	BioLegend	557106
Recombinant mouse Osteopontin	BioLegend	763606
<i>InVivo</i> MAb Anti-mouse/human osteopontin	Bioxcell	BE0372
<i>InVivo</i> MAb mouse IgG2c isotype control	Bioxcell	BE0366
Prednisolone	Pharmaceutical Associates, Inc	NDC 0121-0759-08
Dexamethasone	Sigma-Aldrich	D4902-25MG
L-leucine	Sigma-Aldrich	L-5652



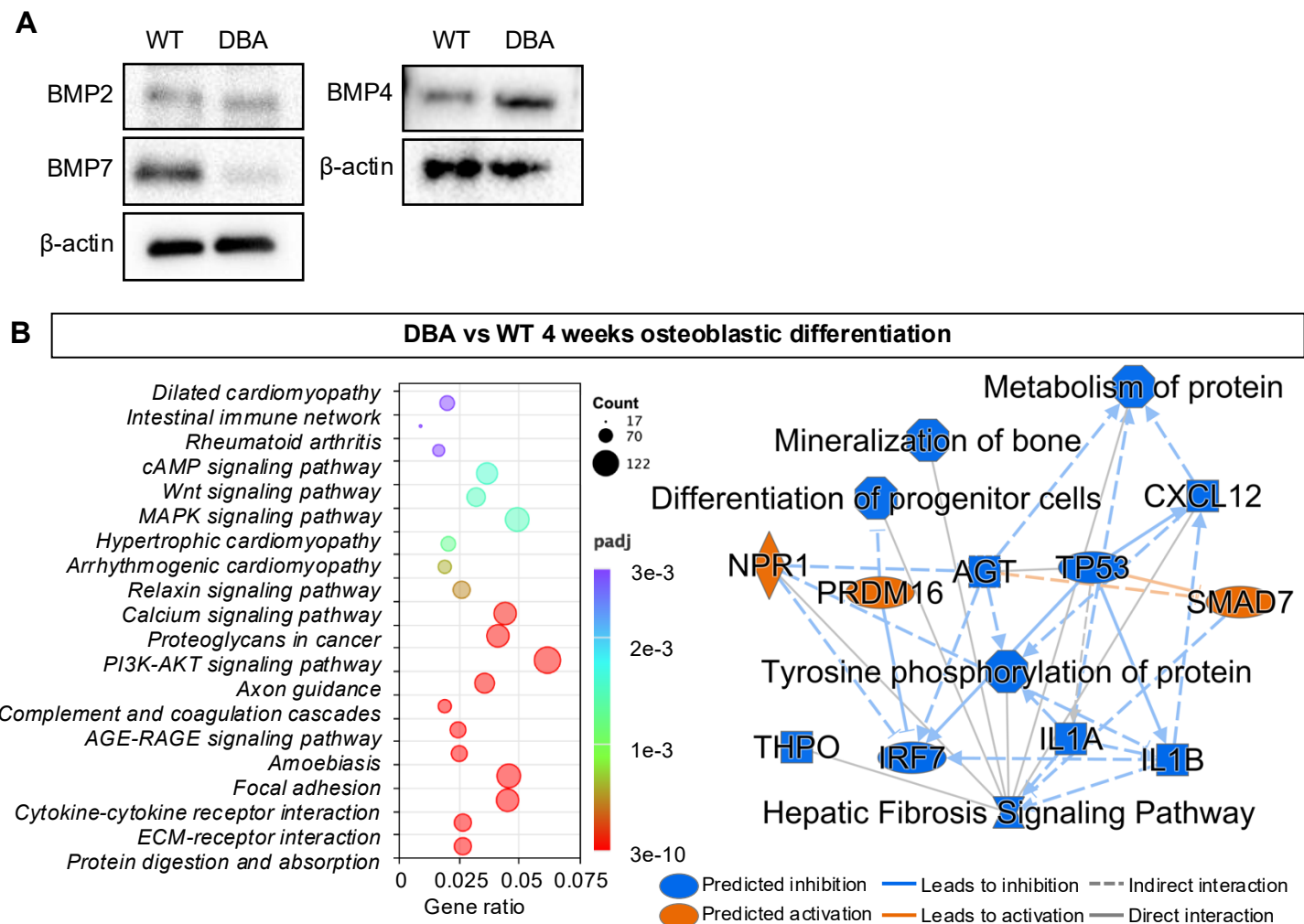
Supplementary Figure 1. Generation of DBA mice-derived MSCs using Cre-ERT2-Rpl11^{+/lox} and Mx-Cre-Rpl11^{+/lox} transgenic mouse models (A) Schematic diagram of generating DBA mouse models using Cre-ERT2 or Mx-Cre strains. Red blood cell (RBC) and hemoglobin values of (B) Cre-ERT2-Rpl11^{+/lox} and (C) Mx-Cre-Rpl11^{+/lox} assessed by complete blood counts (CBC). (D) Schematic diagram describing MSC isolation from mouse bone marrow. (E) Rpl11 protein level in *Ex vivo* expanded WT and DBA MSCs derived from Cre-ERT2 strain. (F) Surface molecule expression of WT and DBA MSCs assessed by flow cytometry. Black lines indicate the positive gate for the marker. (G) Tri-lineage differentiation potential of WT and DBA MSCs was assessed by staining the differentiated cells with Alizarin Red, Red Oil O, and Alcian Blue (from left to right) for identifying osteoblasts, adipocytes, and chondrocytes, respectively. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: *<0.05, **<0.01, ***<0.001, ****<0.0001



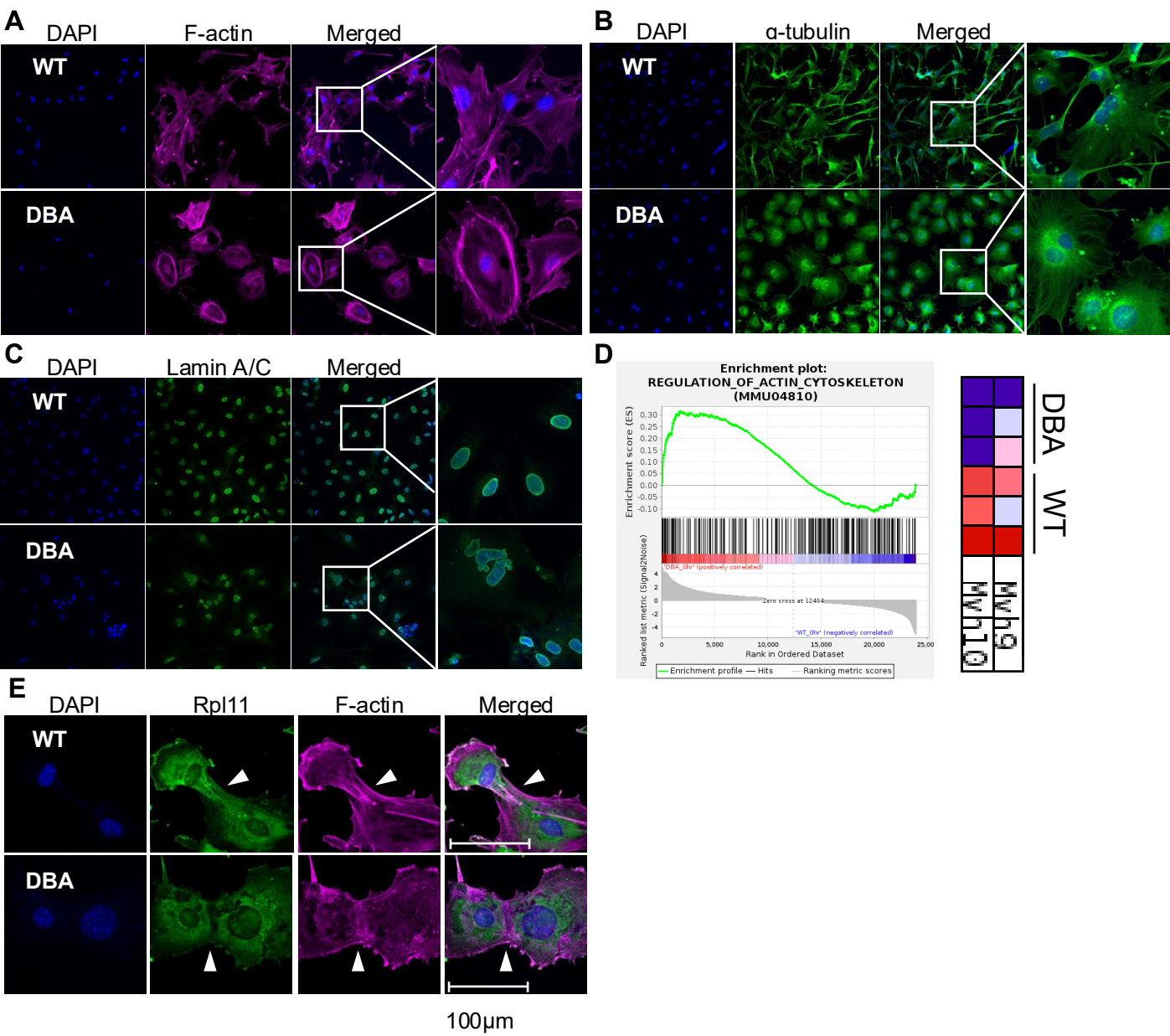
Supplementary Figure 2. Expression of cell cycle-associated proteins and synchronized cell cycle progression in WT and DBA mouse MSCs. (A) Comparison of cell cycle arrest at 24 and 48 hours of serum starvation in WT and DBA MSCs. (B) Timeline of the synchronized cell cycle progression assay. (C) Flow cytometry plot presenting cell cycle progression in WT and DBA MSCs post-release from serum starvation. (D) Expression of G1 and S phase-associated proteins Cyclin E, Cyclin D, and CDK2. (E) mRNA and protein expression of p53 and downstream p21 in WT and DBA MSCs. (F) Representative flow cytometry plots and quantification of apoptosis assessed by Annexin V and 7AAD staining. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001



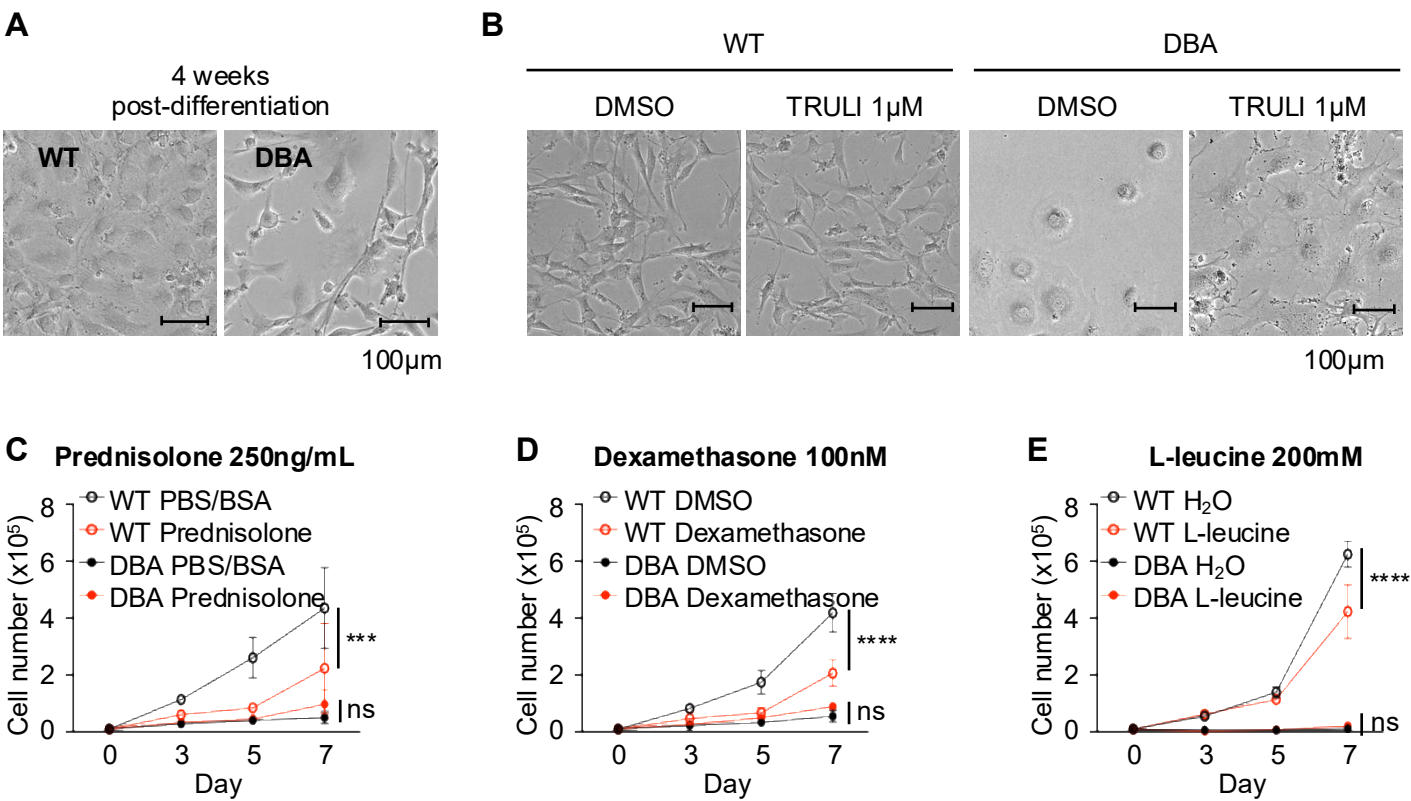
Supplementary Figure 3. Generation of human DBA MSCs (A) Induced pluripotent stem cells (iPSCs) derived from a healthy donor (HD) and a DBA patient with RPL11 mutation. (B) Microscopic images of HD and DBA patient iPSC-derived iMSC on day 1 and 8 of differentiation. (C) Quantification of RPL11 protein expression in human iPSC-derived MSCs. (D) Surface molecule expression of WT and DBA MSCs assessed by flow cytometry. Black lines indicate the positive gate for the marker. (E) Tri-lineage differentiation potential of WT and DBA MSCs was assessed by staining the differentiated cells with Alizarin Red, Red Oil O and Alcian Blue (from left to right) for identifying osteoblasts, adipocytes and chondrocytes, respectively. (F) *RPL11* mRNA, cell proliferation and cell viability of DBA-like patient-derived MSCs. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001



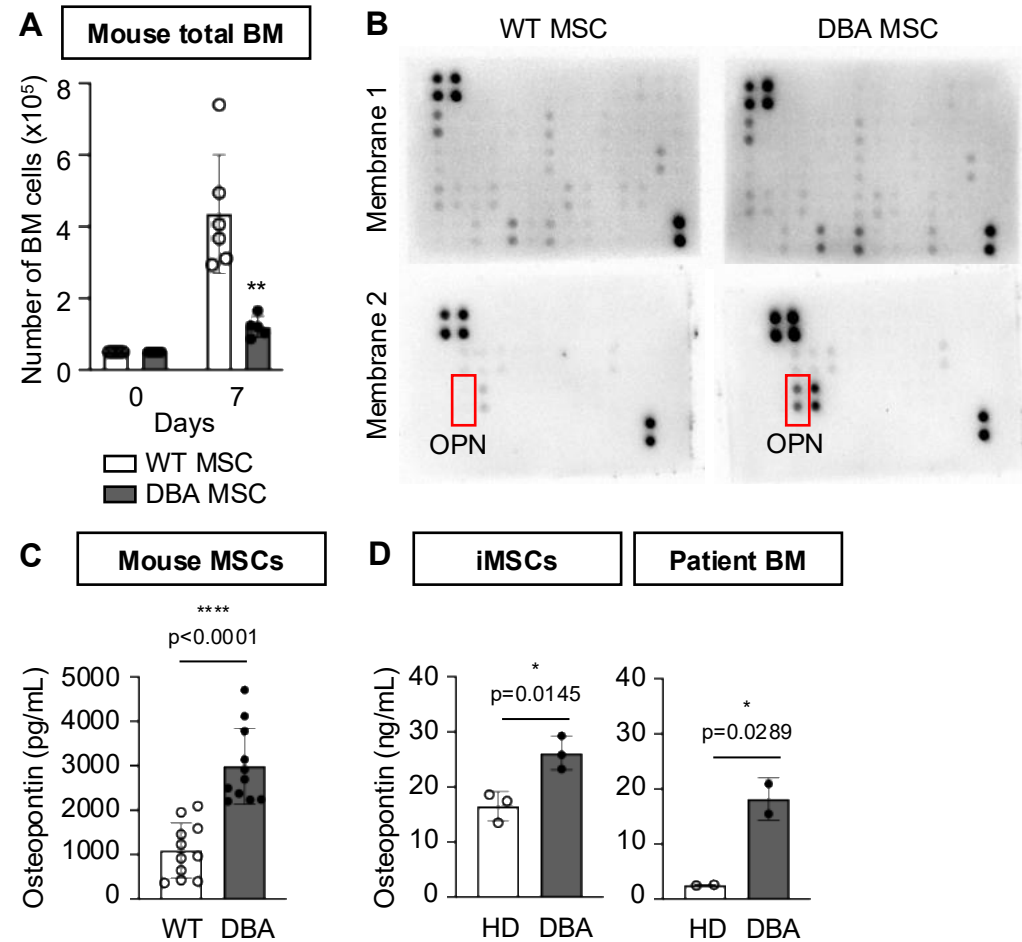
Supplementary Figure 4. Figure 3. Bulk-RNA sequencing of WT and DBA MSCs. (A) Protein expression of BMP2, 4, and 7 in WT and DBA MSCs using western blot analysis. (B) Differentially enriched pathways defined using Kyoto Encyclopedia of Genes and Genomes (KEGG) dataset (left) and predicted networks analyzed using Ingenuity Pathway Analysis (IPA, Qiagen) (right) in DBA MSCs compared to WT MSCs at 4 weeks of osteoblast differentiation. Differentially expressed genes are filtered with the absolute value of $\log_2(\text{foldchange}) > 1$ and $p\text{-adjusted} < 0.05$



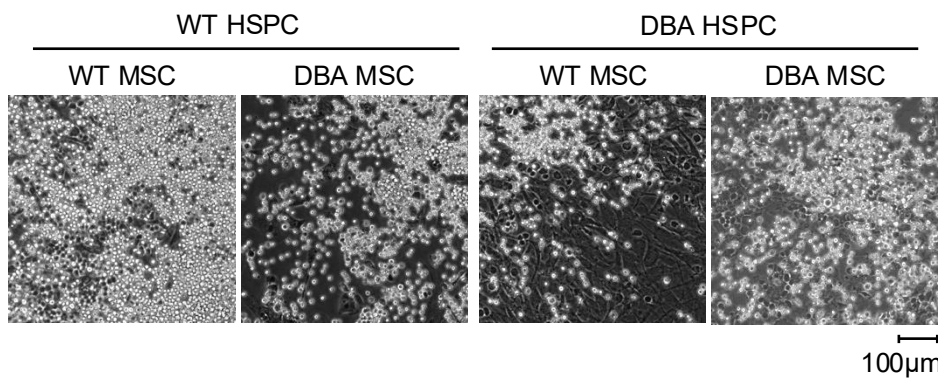
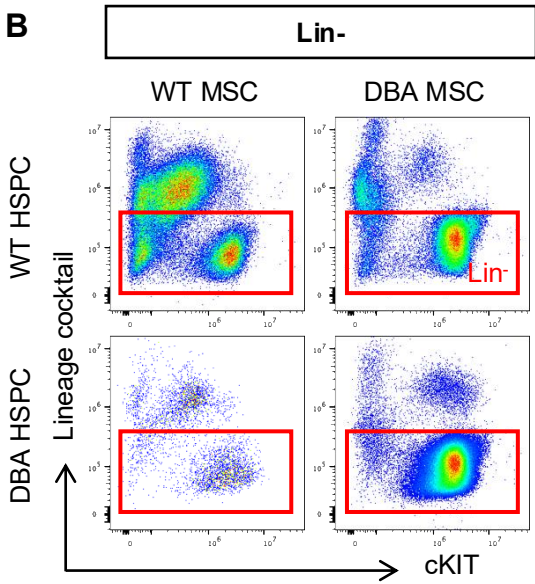
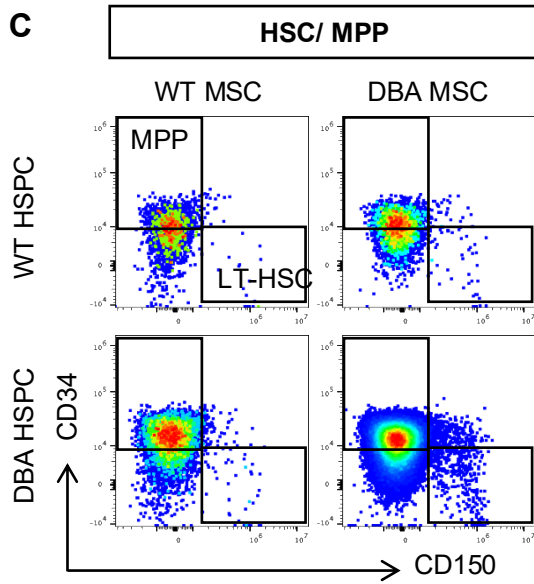
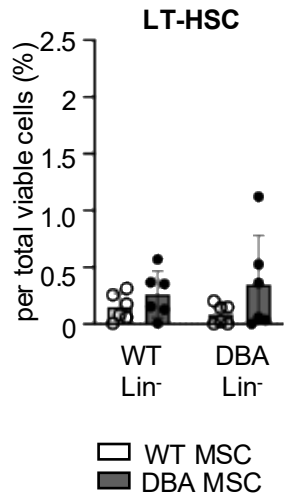
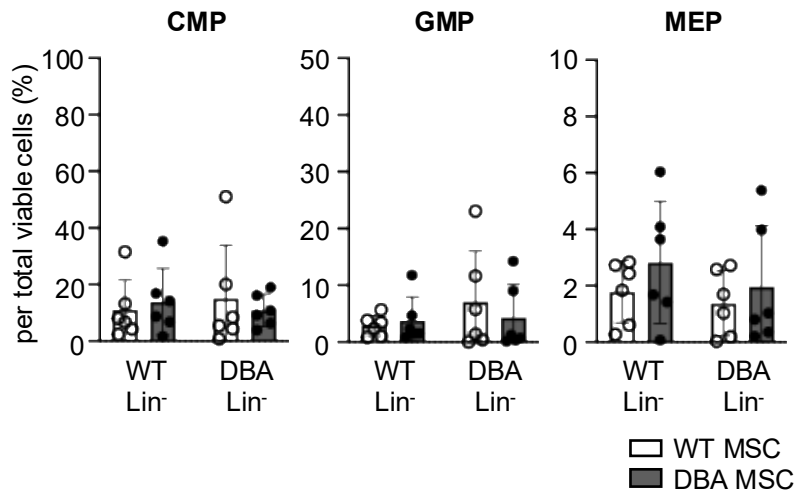
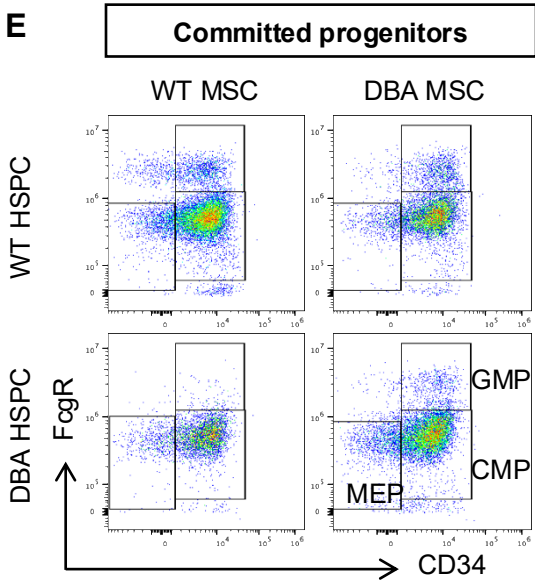
Supplementary Figure 5. Immunofluorescence staining of the cytoskeleton in WT and DBA MSCs. Confocal microscopic images of WT and DBA MSCs stained for (A) F-actin, (B) α -tubulin and (C) Lamin A/C to stain microfilaments, microtubules and intermediate filaments, respectively. (D) Regulation of actin cytoskeleton enrichment plot of undifferentiated DBA MSCs compared to WT MSCs, and Myh9 and Myh10 expression within the gene set. (E) Confocal microscopic images of WT and DBA MSCs during cytokinesis. Cytokinetic events were captured after serum starvation and release into the cell cycle.

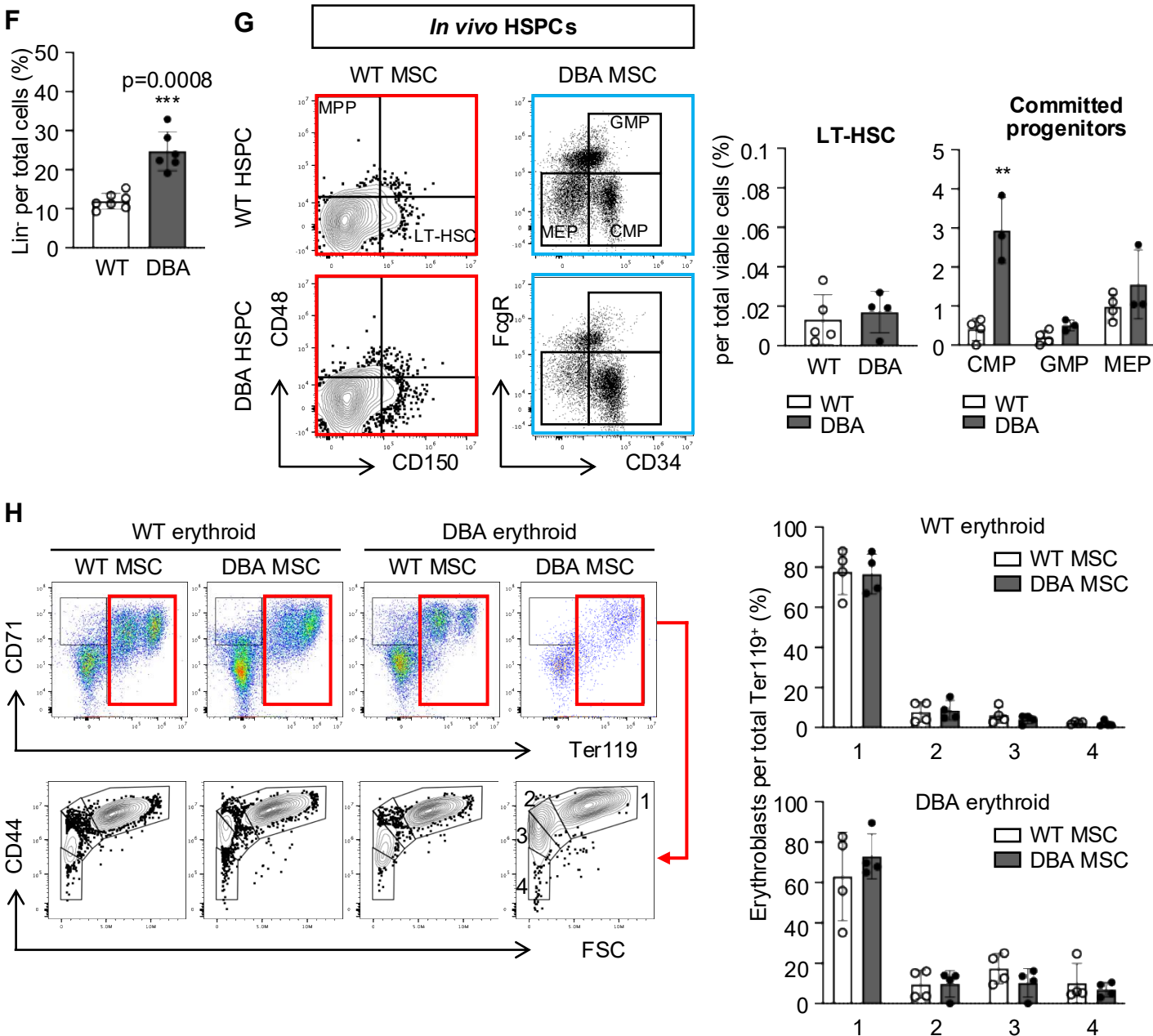


Supplementary Figure 6. Morphological changes of MSCs during osteoblast differentiation and responses to treatments. (A) Microscopic images of mouse WT and DBA MSCs after 4 weeks of differentiation into osteoblasts. (B) Microscopic images of mouse WT and DBA MSCs after 4 weeks of osteoblast differentiation with TRULI treatment. Cell proliferation curves of mouse WT and DBA MSCs treated with (C) prednisolone, (D) Dexamethasone, and (E) L-leucine for 7 days. Cell proliferation was assessed by counting cell numbers using a Trypan Blue exclusion assay. Statistical analysis was performed using two-way ANOVA with a multiple comparisons test. Error bars represent standard deviation (SD). Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001



Supplementary Figure 7. Suppression of healthy HSPC expansion mediated by differential secretion of extrinsic factors by DBA MSCs. (A) Cell counts of total mouse bone marrow cells ($n=6$) after 7 days of culture on healthy ($n=4$) or DBA MSCs ($n=5$). (B) Raw images of the antibody-based secretome array. The red rectangle shows duplicate spots of antibody against osteopontin (OPN). Concentrations of (C) mouse ($n=11$) and (D) human ($n=3$ for iMSC clone 1, $n=3$ for iMSC clone 2, and $n=3$ for bone marrow biopsy-derived MSCs) osteopontin in the cell culture supernatant measured by ELISA. Statistical analysis was performed using Student's t-test comparing the means. Error bars represent standard deviation (SD). p-value: * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001

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Supplementary Figure 8. Hematopoietic trajectory of WT and DBA HSPCs cultured on WT and DBA MSCs using flow cytometry. (A) Microscopic images of mouse WT and DBA HSPCs with WT and DBA MSCs. Bright, white circles are hematopoietic cells, and darker, elongated cells are MSCs. (B) Representative flow cytometry plots showing Lineage-negative populations within the HSPC fraction after 7 days of culture. (C) Representative flow cytometry plots showing long-term hematopoietic stem cells (LT-HSCs) and multipotent progenitors (MPPs). (D) Quantification of LT-HSCs over total viable events. (E) Representative flow cytometry plots and quantification of committed progenitors (CMP (common myeloid progenitors), GMP (granulocyte-monocyte progenitors), and MEP (megakaryocyte-erythroid progenitors) using CD34 and FcγR within the HSPC fraction after 7 days of culture. (F) Quantification of lineage-negative fraction in WT and DBA mice bone marrow. (G) Representative flow cytometry plots and quantification of HSPCs in WT and DBA mice bone marrow. (H) Representative flow cytometry plots and quantification of erythroid maturation of WT and DBA HSPCs co-cultured with WT and DBA MSCs. Statistical analysis was performed using Student's t-test comparing the means between WT and DBA. Error bars represent standard deviation (SD). p-value: *<0.05, **<0.01, ***<0.001, ****<0.0001