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Liver kinase B1 fine-tunes lineage commitment of human fetal synovium-derived stem cells

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Abstract

Liver kinase B1 (LKB1), a serine/threonine protein, is a key regulator in stem cell function and energy metabolism. Herein, we describe the role of LKB1 in modulating the differentiation of synovium-derived stem cells (SDSCs) toward chondrogenic, adipogenic, and osteogenic lineages. Human fetal SDSCs were transduced with CRISPR associated protein 9 (Cas9)-single guide RNA (sgRNA) vectors to knock out or lentiviral vectors to overexpress the *LKB1* gene. Analyses including ICE (Inference of CRISPR Edits) data from Sanger sequencing and qPCR as well as Western blot demonstrated successful knockout (KO) or overexpression (OE) of *LKB1* in human fetal SDSCs without any detectable side effects in morphology, proliferation rate, and cell cycle. *LKB1* KO increased CD146 expression; interestingly, *LKB1* OE increased SSEA4 level. The qPCR data showed that *LKB1* KO up-regulated the levels of *SOX2* and *NANOG* while *LKB1* OE lowered the expression of *POU5F1* and *KLF4*. Furthermore, *LKB1* KO enhanced, and *LKB1* OE inhibited, chondrogenic and adipogenic differentiation potential. However, perhaps due to the inherent inability to achieve osteogenesis, LKB1 did not obviously affect osteogenic differentiation. These data demonstrate that LKB1 plays a significant role in determining human

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AUTHORS' CONTRIBUTIONS

SZ, XBZ, and MP designed the project; SZ, YWF, and XBZ performed the research; SZ, YWF, XBZ, and MP analyzed the data; and SZ and MP wrote the draft. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SDSCs' adipogenic and chondrogenic differentiation, which might provide an approach for fine-tuning the direction of stem cell differentiation in tissue engineering and regeneration.

Graphical Abstract

Human fetal SDSCs (synovium-derived stem cells) were transduced with lentiviral vectors to overexpress *LKB1* (liver kinase B1) or CRISPR associated protein 9 (Cas9)-single guide RNA (sgRNA) vectors to knock out it. Following expansion, transduced human fetal SDSCs were induced for chondrogenic, adipogenic, and osteogenic differentiation. Overexpression of *LKB1* led to decreases in chondrogenic and adipogenic potentials, which were enhanced by knockout of *LKB1*. *LKB1* did not obviously affect osteogenic differentiation.

Keywords

Liver kinase B1; Adipogenesis; Chondrogenesis; Synovium-derived stem cell; CRISPR-Cas9; Lentivirus

Introduction

Liver kinase B1 (*LKB1*), also known as serine/threonine protein kinase 11 (*Stk11*), was first identified as a tumor suppressor mutated in Peutz-Jeghers syndrome.¹ Global knockout (KO) of *LKB1* leads to embryonic lethality, suggesting that *LKB1* is critical for embryonic development.² As a key regulator in stem cell function,³ inhibition of *LKB1* down-regulates differentiation-related genes while up-regulating pluripotency genes in human embryonic stem cells.⁴ In the bone marrow, *LKB1* plays a critical role in hematopoietic stem cell biology, such as regulating cell cycle progression and maintaining stem cell survival and quiescence.⁵ Deletion of *LKB1* in muscle progenitor cells during development results in defective myogenesis and premature postnatal death.³

LKB1 is also involved in the development of cartilage, bone, and adipose tissue.^{6–9} LKB1 plays an essential role in maintaining a balance between mitotic and postmitotic mature chondrocytes during development of the mammalian skeleton.⁶ By regulating the number of osteoblasts directly and osteoclasts indirectly, LKB1 plays a vital role in the development of normally structured bone.⁹ LKB1 also regulates adipogenesis. *FABP4*-Cre mediated adipocyte-specific deletion of *LKB1* leads to reduction in white adipose tissue, while *ADIPOQ*-Cre mediated deletion of *LKB1* promotes brown adipose tissue development.^{7,8} This discrepancy may be due to the leaky expression of *FABP4*-Cre in non-adipose tissues.^{10,11}

The abovementioned basic studies demonstrate an important role of LKB1 in regulating stem cell differentiation. The aim of this study was to evaluate the effects of LKB1 on cell fate determination of human fetal synovium-derived stem/stromal cells (SDSCs) upon differentiation induction. We demonstrated that LKB1 signaling regulates SDSC differentiation, particularly for the chondrogenic and adipogenic lineage, indicating that LKB1 signaling might be harnessed to promote targeted differentiation of stem cells for tissue regeneration.

METHODS

Cloning of knockout and overexpression vectors

The use of VSV-G pseudotyped lentiviral vectors were approved by West Virginia University Institutional Biosafety Committee (IBC) (protocol # 17-10-05). To knock out human *LKB1*, the spacer sequence of single-guide RNA (sgRNA) was cloned into lentiviral backbone pRSC-U6-sgRNA-SFFV-Cas9-Puro-Wpre, in which the U6 promoter drives the expression of sgRNA and the spleen focus-forming virus (SFFV) promoter drives the expression of both CRISPR associated protein 9 (Cas9) and the puromycin-resistant gene. The spacer sequences are as follows: sgLKB1–1, GATTTTGAGGGTGCCACCGG; sgLKB1–2, GTTGCGAAGGATCCCCAACG. The vector cloning and sgRNA design have been detailed elsewhere.^{12,13} The sgLKB1–1 and sgLKB1–2 groups were hereinafter referred as to KO1 and KO2, respectively. All of the insertions and deletions were verified by Sanger sequencing. We used the DNA-calcium phosphate co-precipitation method to package lentiviral vectors. After 100-fold concentration by ultracentrifugation, transducing HT1080 cells were used to determine the biological titers of the vectors. To construct the LKB1 overexpression (OE) vectors, the open reading frame (ORF) sequence of the human *LKB1* gene was cloned into a lentiviral vector backbone pRSC-EF1-X-E2A-Puro-Wpre using a Gibson Assembly® kit purchased from New England BioLabs Inc. (NEB, Ipswich, MA).

Cell culture and lentiviral transduction

Human fetal SDSCs were purchased from ScienCell™ Research Laboratories (Carlsbad, CA).¹⁴ Tissue culture plastic expanded passage 3 fetal SDSCs were plated in T25 cell culture flasks using growth medium containing Alpha Modified Eagle's Medium (α-MEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone. Lentiviral vectors were supplemented at a multiplicity of infection of two

with 8 µg/mL protamine sulfate for a 24-h incubation. Starting day two, puromycin (2 µg/mL) was added in culture medium for a total of four days to select against untransduced cells. To observe the morphology, cells were visualized with a Nikon TE2000-S Eclipse inverted microscope (Melville, NY).

Determining gene expression levels by TaqMan® real-time PCR and Western blot analyses

Total RNAs were extracted from transduced cells using TRIzol® (Life Technologies, Carlsbad, CA). Two µg mRNA were used for reverse transcription with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) at 37°C for 120 min. The primers and probe for analyzing the *LKB1* gene (assay ID: Hs00975986_m1) were purchased from Applied Biosystems. *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase; assay ID: Hs02758991_g1) was used to control the amount of RNA in each assay. TaqMan® real-time polymerase chain reaction (PCR) was conducted with iCycler iQ Multi Color Real-Time PCR Detection system (Perkin-Elmer, Wellesley, MA). Relative transcript levels were calculated as $\chi = 2^{-Ct}$, in which $Ct = E - C$, $E = Ct_{exp} - Ct_{GAPDH}$, and $C = Ct_{ct1} - Ct_{GAPDH}$.

For Western blot analysis, expanded cells were dissolved in protein purification lysis buffer (Cell Signaling Technology, Boston, MA) with protease inhibitors on ice for 30 min. Total protein was quantified using the BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Thirty µg of protein from each sample were denatured and separated using Novex™ WedgeWell™ 4–20% Tris-Glycine Gel in the Novex Mini-Cell (Invitrogen, Carlsbad, CA) at 120 V for 3 h. Separated proteins were transferred onto a nitrocellulose membrane using an XCell II™ Blot module (Life Technologies) at 70 V at 4°C overnight. The membrane was incubated overnight with LKB1 polyclonal antibody (Catalog no. 5132S; Cell Signaling Technology) in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) supplemented with 5% bovine serum albumin (BSA), followed by staining with horseradish peroxidase-conjugated goat anti-mouse (Thermo Fisher Scientific) secondary antibody for 1 h. An antibody against GAPDH was used as a loading control. SuperSignal West Femto Maximum Sensitivity Substrate and CL-XPosure Film (Thermo Fisher Scientific) were used for exposure. Each set of samples was repeated three times.

Determination of LKB1 indel mutations by Sanger sequencing and ICE analysis

Cells from the LKB1 KO group were collected for DNA extraction after Cas9-sgLKB1 transduction using Genomic DNA Extraction Kit (Qiagen, Germantown, MD). The LKB1 sequence was amplified with KAPA HiFi DNA polymerase by PCR using the following primers, sgLKB1-1-F: GAGTGTGCGTGTGGTGAGTG, sgLKB1-1-R: GTGTGCCTGGACTTCTGTGA; sgLKB1-2-F: GAGCTGATGTCGGTGGGTAT; sgLKB1-2-R: CGCTACCAGGGCATTTTAAC. The PCR cycling conditions were 95°C for 4 min followed by 30 cycles of 98°C for 5 sec, 60°C for 5 sec, 68°C for 5 sec, 72°C for 5 sec. Column purified PCR products were used for Sanger sequencing (MCLAB). The indel percentage was determined by the web-based tool ICE (Inference of CRISPR Edits).¹⁵

Analyses of cell cycle and proliferation rate

Cell cycle distribution was analyzed using propidium iodide (PI)/RNase (BD Pharmingen, San Jose, CA) staining. Briefly, 1×10^6 human fetal SDSCs were fixed in 70% ethanol at 4°C overnight. Cells were then permeabilized with cold 0.2% Tween 20 in phosphate buffered saline (PBS) for 15 min at 37°C and incubated with PI/RNase for 15 min at room temperature. Data were analyzed by a FACS Calibur (BD Biosciences, San Jose, CA) using the FCS Express software package (De Novo Software, Los Angeles, CA).

Cell proliferation was measured using the Click-iT™ Edu Alexa Fluor™ 647 Flow Cytometry Assay Kit (Invitrogen). Human fetal SDSCs (5×10^5 cells) were seeded in one T75 flask 24 h followed by incubation in fresh growth medium or growth medium containing 10 μM 5-ethynyl-2'-deoxyuridine (Edu). Cells cultured without Edu were used as a negative control. Cells were incubated for 18 h followed by trypsinization and subjected to Edu staining according to the manufacturer's protocol. Briefly, cells were washed with 100 μL of 1% BSA-PBS, incubated with 100 μL Click-iT™ fixative for 15 min at room temperature in the dark, washed with 200 μL 1% BSA-PBS, resuspended with 100 μL $1 \times$ Click-iT™ saponin-based permeabilization and wash reagent (component E), stained in labeling cocktail for 30 min, washed with 500 μL $1 \times$ component E, and analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software).

Evaluation of cell surface phenotypes and stemness genes

The following primary antibodies were used to assess human fetal SDSC surface phenotypes: anti-human CD146-PE (eBioscience, San Diego, CA), anti-human SSEA4 (the stage-specific embryonic antigen 4)-PE (BioLegend, San Diego, CA), and isotype-matched IgGs (Beckman Coulter, Indianapolis, IN). 5×10^5 cells were incubated on ice in PBS containing 0.1% Chrom-Pure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.1% NaN₃ (Sigma-Aldrich, St. Louis, MO) for 30 min. Cells were incubated for 30 min in the dark with the primary antibodies. Fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software).

For quantitative real-time PCR (qPCR), total RNA was extracted from expanded cells in TRIzol® (Life Technologies). Two μg mRNA were used for reverse transcription with the High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Primers and probes for analyzing stemness genes such as *SOX2* (SRY-box 2; assay ID: Hs01053049_s1), *NANOG* (assay ID: Hs02387400_g1), *POU5F1* (POU domain, class5, transcription factor 1; assay ID: Hs04260367_gH), *KLF4* (Kruppel-like factor 4; assay ID: Hs00358836_m1), *BM11* (B lymphoma Mo-MLV insertion region 1 homolog; assay ID: Hs00180411_m1), *MYC* (assay ID: Hs00153408_m1), and *NOV* (nephroblastoma overexpressed; assay ID: Hs00159631_m1), were purchased from Applied Biosystems as part of their Custom TaqMan® Gene Expression Assays. *GAPDH* was used as the endogenous control gene. All reactions were conducted in triplicate.

Tri-lineage differentiation

Chondrogenic differentiation—Expanded human fetal SDSCs (3×10^5) were centrifuged at 1200 rpm for 7 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation, the pellets were cultured in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 40 $\mu\text{g/mL}$ proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 0.25 $\mu\text{g/mL}$ fungizone, 0.1 mM L-ascorbic acid-2-phosphate, and 1 $\times$ ITS™ Premix (Corning, Bedford, MA) with the supplementation of 10 ng/mL transforming growth factor beta 3 (TGF- β 3, PeproTech, Rocky Hill, NJ) in a 5% O₂ incubator for up to 28 days. The pellets were assessed using Alcian blue staining for sulfated glycosaminoglycans (GAGs) and immunohistochemical staining (IHC) for type II collagen. For qPCR, one μg mRNA was used for reverse transcription and chondrogenic marker genes were evaluated [*SOX9* (SRY-Box 9; assay ID: Hs00165814_m1), *COL2A1* (type II collagen; assay ID: Hs00156568_m1), and *ACAN* (aggrecan; assay ID: Hs00153935_m1)]. *GAPDH* was carried out as the endogenous control gene. All reactions were conducted in triplicate.

Representative pellets ($n = 3$) were immersed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating with a gradient ethanol series, clearing in xylene, and embedding in paraffin blocks. Five μm sections were stained with Alcian blue staining (counterstained with fast red) for sulfated GAGs. For IHC, the sections were immunolabeled with a primary antibody against type II collagen [II-II6B3, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA], followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,30-diaminobenzidine as a substrate.

Adipogenic differentiation—Expanded cells were seeded at 8000 cells/cm² and cultured for 21 days in adipogenic medium (growth medium supplemented with 1 μM dexamethasone, 0.5 mM isobutyl-1-methyloxanthine, 200 μM indomethacin, and 10 μM insulin). Induced cells in T25 flasks ($n=3$) were fixed in freshly prepared 4% paraformaldehyde and stained with 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 10 min. Lipid droplet bound staining was photographed under a Nikon TE300 phase contrast microscope (Nikon). For qPCR, two μg mRNA were used for reverse transcription and adipogenic marker genes were evaluated [*LPL* (lipoprotein lipase; assay ID: Hs00173425_m1) and *CEBPA* (CCAAT enhancer binding protein alpha; assay ID: Hs00269972_s1)]. *GAPDH* was carried out as the endogenous control gene. All reactions were conducted in triplicate.

Osteogenic differentiation—Expanded cells were seeded at 8000 cells/cm² and cultured for 21 days in osteogenic medium (growth medium supplemented with 0.01 μM dexamethasone, 10 mM β -glycerol phosphate, and 50 mg/L ascorbate-2-phosphate) with or without 50 ng/mL bone morphogenetic protein 2 (BMP2) (PeproTech). Human bone marrow stromal cells (BMSCs) obtained from Lonza Group Ltd. (Basel, Switzerland)¹⁶ were used as a positive control. To evaluate calcium deposition, induced cells were fixed in 4% paraformaldehyde at room temperature for 1 h followed by incubation in 40 mM Alizarin Red S (ARS) solution for 20 min with agitation. After rinsing with PBS, matrix mineral

bound staining was photographed. For qPCR, two µg mRNA were used for reverse transcription and osteogenic marker genes were evaluated [*RUNX2* (runt-related transcription factor 2; assay ID: Hs00231692_m1), *SPPI* (secreted phosphoprotein 1; assay ID: Hs00959010_m1), *SP7* (Osterix; assay ID: Hs01866874_s1), and *BGLAP* (bone gamma-carboxyglutamate protein; assay ID: Hs01587814_g1)]. *GAPDH* was used as the endogenous control gene. All reactions were conducted in triplicate.

Statistical analysis

Due to small sample size and uncertainty about the normality of the measurement, the Mann-Whitney Test was utilized to determine if the 0.05 level of significance was achieved. Inferences from the Mann-Whitney Test relate to the medians of the respective distributions instead of means. All calculations were performed using JMP/Pro13.2 software (SAS Institute, Cary, NC).

RESULTS

Lentiviral vector-mediated knockout or overexpression of *LKB1*

We transduced human fetal SDSCs with lentiviral vectors that express Cas9 and sgLKB1–1 (KO1) or sgLKB1–2 (KO2) for gene knockout, and LKB1 ORF for *LKB1* OE. The two CRISPR-Cas9 vectors were guided by sgRNA sequences targeting different LKB1 exon sequences. Both KO and OE groups were selected *via* puromycin supplementation. As 20nt sgRNAs are preferable when genome editing in stem cells, we chose 20nt sgLKB1s to knockout *LKB1* in SDSCs.¹³ A scramble sgRNA sequence was used to serve as a negative control. A successful transduction of vectors was also verified in expanded cells at the mRNA level using qPCR or at a protein level using Western blot (Fig. 1A/B). During the expansion period, *LKB1* KO1 and KO2 exhibited a 50% decrease in *LKB1* mRNA compared to the control (CTRL) group, whereas *LKB1* OE induced a 100-fold greater *LKB1* mRNA expression (Fig. 1A). To characterize the indels after transduction with Cas9-sgLKB1, genomic DNA flanking the target sequences was PCR-amplified and the PCR products were conducted for Sanger sequencing (Fig. 1C). The indel percentage was about 80% in the KO1 group and 91% in the KO2 group. Similar to previous studies, most indels in this study were deletions (Fig. 1C). These data demonstrate that sgLKB1s induced considerable gene disruptions in human fetal SDSCs.

LKB1 affects the expression of stemness genes and surface markers CD146 and SSEA4, but not cell cycle or proliferation rate

To identify whether *LKB1* could influence the surface marker, cell cycle, proliferation, and stemness genes in expanded human fetal SDSCs, flow cytometry analyses of CD146, SSEA4, relative EdU incorporation, PI stained human fetal SDSCs and qPCR analysis of stemness genes (*SOX2*, *NANOG*, *POU5F1*, *KLF4*, *BM11*, and *MYC*) were utilized. Firstly, under an inverted microscope, cells in the KO and OE groups exhibited similar cell morphology and cell density to the CTRL group (Fig. 2A). The flow cytometry analysis showed that, in the KO groups, CD146 was up-regulated whereas SSEA4 was down-regulated, as evidenced by changes in median fluorescence intensity (median). In the OE group, SSEA4 was up-regulated whereas CD146 was slightly up-regulated in median (Fig.

2B/C). The flow cytometry data also showed that the KO and OE groups had a similar percentage of cells in the G₂+S phases and proliferation rate to the CTRL group (Fig. 2D/E). The qPCR data showed that *LKB1* KO significantly increased expression levels of *SOX2* and *NANOG*, while *LKB1* OE lowered expression of *POU5F1* and *KLF4* (Fig. 2F).

***LKB1* knockout promotes and overexpression inhibits chondrogenic differentiation**

The qPCR data demonstrate that, during chondrogenic induction, the levels of *LKB1* mRNA decreased and the efficiencies of *LKB1* KO and OE were maintained (Fig. 3A), which indicates that LKB1 signaling might be involved in chondrogenesis. In expanded cells, interestingly, the KO groups had a higher level of *SOX9* expression and lower levels of *ACAN* expression compared to the CTRL group (Fig. 3B/D). After 28-day chondrogenic induction, *ACAN*, *SOX9*, and *COL2A1* expression were significantly increased in the KO *versus* CTRL groups and dramatically decreased in the OE group (Fig. 3B/C/D). *LKB1* KO yielded 28-day pellets with a larger size than the CTRL group, but *LKB1* OE significantly decreased the size of 28-day pellets (Fig. 3E). Furthermore, the KO group displayed the most intensity of sulfated GAGs as evidenced by Alcian blue staining and type II collagen by immunostaining, followed by the CTRL group with the OE group showing the least intensity (Fig. 3E). These results demonstrate that LKB1 inhibits chondrogenesis and *LKB1* KO increases the chondrogenic potential of human fetal SDSCs.

***LKB1* knockout promotes and overexpression inhibits adipogenic differentiation**

The qPCR data suggest that the efficiencies of *LKB1* KO and OE were maintained during adipogenic differentiation (Fig. 4A). Oil Red O staining data showed that, compared to the CTRL and *LKB1* OE groups, adipogenically induced SDSCs in the *LKB1* KO group exhibited intensive density of lipid droplets staining (Fig. 4B). After lentiviral transduction and cell expansion, *LKB1* KO increased expression of adipogenic markers like *CEBPA* and *LPL* while *LKB1* OE showed no differences (Fig. 4C/D). After a 21-day adipogenic induction, the KO group maintained greater *CEBPA* and *LPL* expression compared with the CTRL group while the OE group had decreased expression of these adipogenic markers (Fig. 4C/D). These data indicate that LKB1 attenuates adipogenic differentiation of human fetal SDSCs.

Effect of *LKB1* KO and OE on osteogenic differentiation

The qPCR data suggest that the efficiencies of *LKB1* KO and OE were maintained during osteogenic differentiation (Fig. 5A). Alizarin Red S staining data suggested that, compared to human BMSCs, human fetal SDSCs had undetectable staining in calcium deposition (Fig. 5B), which was also confirmed by our qPCR data (Fig. 5C–F). During cell expansion, *BGLAP* was down-regulated in the KO group but up-regulated in the OE group. After osteogenic induction, the KO group had lower expression of *BGLAP* and *SP7* in osteogenic treatments with or without BMP2; however, lower expression of *SPP1* occurred only with BMP2 treatment. Interestingly, the OE group exhibited either lower or comparable expression of tested osteogenic marker genes to the CTRL group (Fig. 5C–F).

DISCUSSION

In this study, we found that *LKB1* affects expression of mesenchymal stem cell (MSC) associated surface markers such as CD146 and SSEA4, and stemness related genes such as *SOX2* and *NANOG*. However, no significant changes in proliferation rate and cell cycle were observed. In addition, our KO and OE data demonstrate that *LKB1* negatively affects SDSC commitment to chondrogenic and adipogenic lineages, but has no obvious effects on osteogenic differentiation.

In accordance with a recent hypothesis that there is crosstalk between chondrogenesis and adipogenesis,¹⁷ in this study, we found that the mRNA level of *LKB1* decreased during chondrogenic or adipogenic differentiation of human fetal SDSCs, indicating that *LKB1* might be involved in the determination of these two lineages. To confirm the role of *LKB1* in the differentiation of MSCs, human fetal SDSCs were cultured in various differentiation medium following the KO or OE of *LKB1*. The qPCR results of each marker gene revealed that *LKB1* KO accelerated chondrogenic and adipogenic differentiation through promoting lineage-determining transcription factors, but did not have any obvious influence on osteogenesis. As expected, the opposite effects were observed in the *LKB1* OE group.

In a pellet culture system, *LKB1* KO enhanced chondrogenic differentiation while *LKB1* OE inhibited the chondrogenic process, which may be attributed to its regulation of SOX9, a key transcription factor for chondrogenesis that is also involved in the synthesis of aggrecan and type II collagen.¹⁸ As one of the downstream signals of *LKB1*,¹⁹ AMP-activated protein kinase (AMPK) activity can directly decrease the expression of *SOX9*.²⁰ Suppression of AMPK by small interfering RNA (siRNA) or high-glucose culture conditions promoted the expression of chondrogenic marker genes in both chondrogenic cell line ATDC5 cells and primary mouse embryonic limb cells.²⁰ Conversely, metformin, an activator of AMPK, inhibited the chondrogenic differentiation process.²⁰ Further studies are needed to clarify whether *LKB1* signaling influences chondrogenesis through AMPK activity.

Similarly, we found that *LKB1* KO increased adipogenic differentiation of human fetal SDSCs and *LKB1* OE slowed the adipogenic process. The influence of *LKB1* on adipogenesis was probably associated with *CEBPA* expression, a transcription factor for adipogenesis. Interestingly, *LKB1* KO increased the expression of *LPL* and *CEBPA* even during the expansion period. Our finding is consistent with a study demonstrating that *LKB1* signaling keeps preadipocytes in an undifferentiated stage²¹ despite an *in vivo* study showing that mice lacking *LKB1* in adipose tissue have impaired adipogenesis.⁸ This discrepancy might be due to different stages of adipogenesis that were assessed in these studies. The former study intervened in the preadipocyte stage, while the latter focused on the late stages of adipocyte differentiation. In line with our finding, many other studies have demonstrated that *LKB1* signaling inhibits the adipogenic process.^{22–24} Moreover, deletion of *LKB1* in proliferating myoblasts enhances lipid accumulation while inhibiting myogenic differentiation, suggesting that a deficiency of *LKB1* promotes adipogenic differentiation of myoblasts at the expense of myogenic differentiation.²⁵

It is well known that *SOX2*, *NANOG*, and *POU5F1* are pluripotency markers that play crucial roles in the maintenance of identity and differentiation of pluripotent cells including embryonic stem cells.²⁶ Interestingly, increasing evidence indicates that these markers are also important for MSC differentiation;^{27,28} however, the current outcome was controversial,²⁹ which deserves further investigation. In this study, *LKB1* KO not only promoted chondrogenesis and adipogenesis but also increased expression of *SOX2*, *NANOG*, *POU5F1* and *KLF4*, while *LKB1* OE lowered the expression of *POU5F1* and *KLF4*. These results are in agreement with previous reports in which *LKB1* KO in human amniotic epithelial cells grown on mouse embryonic fibroblasts (hESCs^{MEF}) resulted in up-regulation of pluripotency marker genes *NANOG* and *POU5F1*,^{4,30} while some stemness genes also exhibited a correlation with lineage determination.^{31–37} For instance, *SOX2* inhibition resulted in an increase of chondrogenesis in human umbilical cord blood-derived MSCs³¹ or a decrease in amniotic fluid-derived MSCs.³² Overexpressing *NANOG* and/or *POU5F1* improved chondrogenesis in human BMSCs³³ and human dental pulp stem cells.³⁴ Moreover, lentiviral vector-mediated overexpression of *KLF4* enhanced chondrogenic differentiation in equine chondrocytes.³⁵ In regard to adipogenesis, inhibition of *SOX2* led to a decrease of differentiation in human umbilical cord blood-derived MSCs³¹ while overexpressing *NANOG* or *POU5F1* improved adipogenesis in human BMSCs.³³ Interestingly, co-OE of *POU5F1/SOX2* or *POU5F1/NANOG* enhanced the differentiation in human adipose tissue-derived MSCs³⁶ and human dental pulp stem cells,³⁴ respectively. Moreover, *KLF4* KO inhibited adipogenesis.³⁷ Taken together, higher expression of some unique stemness genes is beneficial for chondrogenic or adipogenic differentiation, which might explain the higher chondrogenic and adipogenic capacity in the *LKB1* KO groups.

The relationship between the level of CD146 or SSEA4 and lineage differentiation is debatable. CD146+ chondroprogenitors in late-stage osteoarthritis or skeletal stem cells from the growth plate exhibited higher chondrogenic differentiation capacity.^{38,39} Fluorescence activated enrichment of CD146+ human BMSCs during expansion augmented GAG/DNA content but had no effect on adipogenesis.⁴⁰ Another study found that CD146+ human placenta-derived MSCs had identical chondrogenic or adipogenic differentiation potential compared with the CD146- cells.⁴¹ However, *CD146* KO impaired adipogenic differentiation in 3T3-L1 fibroblasts.⁴² The level of SSEA4 in human BMSCs or Wharton's Jelly-derived MSCs did not correlate with either chondrogenic⁴³ or adipogenic potential.⁴⁴ In our study, *LKB1* KO cells showed higher CD146 and lower SSEA4 expression, which are associated with enhanced chondrogenic and adipogenic differentiation. The relationships between CD146 or SSEA4 level and differentiation of MSCs needs to be further investigated.

Surprisingly, we did not observe obvious effects of *LKB1* KO and OE on osteogenesis of human fetal SDSCs. One possibility is that SDSCs are a kind of stem cell inherently favoring chondrogenesis and adipogenesis rather than osteogenesis.^{45,46} Moreover, the precise role of *LKB1* depends on cell types and different stages. For example, activation of the *LKB1/AMPK* pathway was associated with metformin-induced osteoblastic differentiation of human induced pluripotent stem cell-derived MSCs;⁴⁷ however, conditional deficiency of *LKB1* in osteoblast precursors resulted in increased osteoblast number and activity.⁹

In conclusion, *LKB1* KO can modulate the lineage differentiation of SDSCs toward chondrogenesis and adipogenesis. These data indicate that LKB1 signaling may be harnessed to fine-tune the direction of stem cell differentiation for cartilage and adipose regeneration.

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Fig 1.

Successful KO and OE of *LKB1*. Human fetal SDSCs were transduced with scramble sgRNA sequence-containing vector (Control or CTRL), CRISPR-Cas9 vectors (sgLKB1-1 or KO1 and sgLKB1-2 or KO2), or *LKB1* the open reading frame (ORF) vector (OE). Following transduction, LKB1 expression was measured by qPCR (A) and Western blot (B) in cell samples. GAPDH served as an endogenous control. Data are shown as bar charts. * indicates a significant difference compared to the CTRL ($p < 0.05$). DNA sequences of indel mutations induced by sgLKB1 in human fetal SDSCs (C).

Fig 2.

Evaluation of cell morphology, surface markers, proliferation rate, and stemness genes in human fetal SDSCs after KO and OE of *LKBI*. (A) Morphological appearance of monolayer cultures was observed at passage 4. Flow cytometry was used to measure both median and percentage of surface markers (CD146 and SSEA4) (B/C), relative EdU incorporation (D), and the percentage of cells in the G₂+S phases (E) in the CTRL, KO1, KO2, and OE groups. qPCR analysis of stemness genes (*SOX2*, *NANOG*, *POU5F1*, *KLF4*, *BMI1*, and *MYC*) was performed in all groups with the CTRL group of *POU5F1* expression level being set to one (F). Data are shown as bar charts. * indicates a significant difference compared to the corresponding CTRL ($p < 0.05$).

Fig 3.

Evaluation of chondrogenic potential after KO and OE of *LKB1*. Human fetal SDSCs were chondrogenically induced in a pellet culture system for 28 days. The transduction efficiency of *LKB1* was maintained during induction (A). The effect of *LKB1* on chondrogenic capacity of human fetal SDSCs was evaluated using qPCR for chondrogenic marker gene expression (*SOX9*, *COL2A1*, and *ACAN*) (B-D) and Alcian blue staining for sulfated GAGs and immunohistochemical staining (IHC) for type II collagen (E). *GAPDH* was carried out as an endogenous control. Data are shown as bar charts. * indicates a significant difference compared to the corresponding CTRL ($p < 0.05$).

Fig 4.

Evaluation of adipogenic potential after KO and OE of *LKB1*. Human fetal SDSCs were adipogenically induced in differentiation medium for 21 days. The transduction efficiency of *LKB1* was maintained during induction (A). The effect of *LKB1* on adipogenic capacity of human fetal SDSCs was evaluated using Oil Red O staining to observe lipid droplets produced by induced SDSCs (B) and qPCR for adipogenic marker gene expression (*CEBPA* and *LPL*) (C/D). *GAPDH* was carried out as an endogenous control. Data are shown as bar charts. * indicates a significant difference compared to the corresponding CTRL ($p < 0.05$).

Fig 5.

Evaluation of osteogenic potential after KO and OE of *LKB1*. Human fetal SDSCs were osteogenically induced in differentiation medium for 21 days. The transduction efficiency of *LKB1* was maintained during induction (A). The effect of *LKB1* on osteogenic capacity of human fetal SDSCs was evaluated using Alizarin Red S staining to identify calcium deposition of induced SDSCs (B) and qPCR for osteogenic marker gene expression (*BGLAP*, *SPP1*, *RUNX2*, and *SP7*) (C-F). *GAPDH* was carried out as an endogenous control. Data are shown as bar charts. * indicates a significant difference compared to the corresponding CTRL ($p < 0.05$).