Ascorbate and Iron Are Required for the Specification and Long-Term Self-Renewal of Human Skeletal Mesenchymal Stromal Cells

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SUMMARY

The effects of ascorbate on adult cell fate specification remain largely unknown. Using our stepwise and chemically defined system to derive lateral mesoderm progenitors from human pluripotent stem cells (hPSCs), we found that ascorbate increased the expression of mesenchymal stromal cell (MSC) markers, purity of MSCs, the long-term self-renewal and osteochondrogenic capacity of hPSC-MSCs in vitro. Moreover, ascorbate promoted MSC specification in an iron-dependent fashion, but not in a redox-dependent manner. Further studies revealed that iron synergized with ascorbate to regulate hPSC-MSC histone methylation, promote their long-term self-renewal, and increase their osteochondrogenic capacity. We found that one of the histone demethylases affected by ascorbate, KDM4B, was necessary to promote the specification of hPSC-MSCs. This mechanistic understanding led to the metabolic optimization of hPSC-MSCs with an extended lifespan in vitro and the ability to fully repair cartilage defects upon transplantation in vivo. Our results highlight the importance of ascorbate and iron metabolism in adult human cell fate specification.

INTRODUCTION

Ascorbate, or vitamin C, is an important cofactor for many biochemical reactions in the human body, and thus exerts pleiotrophic effects in stem cell biology. For example, ascorbate can function as a potent antioxidant, a cofactor for dioxygenases, and a cofactor for collagen synthesis. Although the effects of ascorbate on induced pluripotent stem cell (iPSC) reprogramming and on iPSCs are well known (Chen et al., 2013; Chung et al., 2010; Wang et al., 2011), its effects on adult cell fate specification have been less clear.

Embryonic mesoderm progenitors, under the influence of a multitude of developmental signaling pathways, produce the long bones in the adult limbs, which also harbor adult skeletal mesenchymal stromal cells (MSCs) (Alman, 2015; Prockop, 2009). During embryonic development, all cartilage, bone, and bone marrow in the limbs and the appendicular skeleton originate from the lateral plate mesoderm. Adult skeletal MSCs are capable of multi-lineage differentiation into cartilage, bone, and adipose tissues. However, both scientific and clinical studies of MSCs have been hampered by their poor retention of differentiation potential and self-renewal capacity during long-term culture in vitro. Although there are reports of MSCs derived from the infinitely self-renewing human pluripotent stem cells (hPSCs) (Barberi et al., 2005; Hwang et al., 2008; Olivier et al., 2006; Vodyanik et al., 2010), their use of non-defined components, such as fetal bovine serum or OP9 feeder cells, greatly compromise their consistency and clinical applicability. Current differentiation protocols of iPSCs into MSCs are also not efficient, often requiring cell sorting. Thus, there is a need to establish a chemically defined platform to fully recapitulate each major phase of MSC development efficiently. Such a platform would also be useful to test the roles of ascorbate and other small molecules during skeletal MSC specification and long-term self-renewal.

In this study, we developed a platform to fully recapitulate the major phases of MSC development in vitro. This in vitro platform involves three phases, including the induction of primitive streak cells, differentiation into lateral mesoderm progenitors, and specification of MSCs. This platform provides intermediate cells, previously inaccessible in human embryos, that represent the different phases of MSC development. In the process, we found that ascorbate increased the
expression of MSC markers by transcriptomic profiling, increased the purity of MSCs by surface antigen profiling, and increased the self-renewal and osteochondrogenic capacity of hPSC-MSCs. Moreover, ascorbate promoted MSC specification in an iron-dependent fashion, but not in a redox-dependent manner. Further studies revealed that iron synergized with ascorbate to regulate histone methylation in hPSC-MSCs, promote their self-renewal and increase their osteochondrogenic capacity. Furthermore, our results suggest that one of the JmjC histone demethylases affected by ascorbate, KDM4B, is necessary and sufficient to promote specification of lateral mesoderm progenitors into human MSCs. This mechanistic understanding led to the derivation of human MSCs with an extended lifespan and enhanced osteochondrogenic potential. Furthermore, our hPSC-MSCs can fully repair cartilage defects upon transplantation in vivo. These results highlight the importance of ascorbate and iron metabolism in adult stem cell fate specification and long-term self-renewal, with important implications for tackling human stem cell aging (Li et al., 2016; Zhang et al., 2015).

RESULTS

Optimal Activin and Wnt Synergism for Complete Induction of Primitive Streak Cells

iPSCs were generated from MRC5 human embryonic lung fibroblasts or BJ foreskin fibroblasts (Figure S1) (Takahashi et al., 2007). Activin A is known to be essential to induce primitive streak cells from PSCs (Gadue et al., 2006; Nostro et al., 2008). Our results showed that activin A enhanced induction into primitive streak cells expressing the key transcription factors (TFs): T (Brachyury) and MIXL1 (Figure 1A). However, the endodermal TF SOX17 was also increased with increasing doses of activin A. We found that the ratio of MIXL1 (or T) to SOX17 was the highest when we optimized the dose at 25 ng/mL of activin A (Figure 1B). Wnt signaling is also essential for inducing primitive streak cells from PSCs (Gadue et al., 2006; Liu et al., 1999). CHIR99021, a GSK3β inhibitor, is known to activate canonical Wnt signaling by stabilizing β-catenin. Our data showed that activin A and CHIR99021 synergistically promoted primitive streak induction. Compared to Wnt3a, CHIR99021 was superior in promoting cell adherence (Figure S2A), as well as induction of the primitive streak TFs: MIXL1, T (Brachyury), and GSC (Figure S2B). Although addition of fibroblast growth factor 2 (FGF2) at day 2 did not further enhance primitive streak induction, expression of mesoderm TFs, such as HAND1 and FOXF1, increased in the presence of FGF2 (Figure S2B).

Thus, in phase 1 (D0-2) of our platform (Figure S2A), i.e., primitive streak induction, SOX2 significantly decreased, while the primitive streak TFs T (Brachyury), MIXL1, and GSC peaked at day 2 (Figures 1C and S3). Fluorescence-activated cell sorting (FACS) showed that our protocol yielded 98.13% ± 1.7% T+, 97.53% ± 0.7% MIXL1+, and 98.77% ± 1.13% GSC+ cells (Figure 1D). Immunofluorescence staining confirmed the qRT-PCR and FACS data (Figure 1E). Several mesoderm markers, such as WNT3, PDGFRα, PDGFRβ, HAND1, and FOXF1 were also upregulated. In contrast, endodermal TFs, such as SOX17, FOXA1, FOXA2, and ectodermal TFs, such as SOX2 and PAX6 were either downregulated or remained low in expression (Figures 1C, S2B, and S3). Genome-wide epigenetic patterns were consistent with gene expression. Chromatin immunoprecipitation sequencing (ChIP-seq) for methylation of histone H3 Lys 4 (H3K4me3) and H3 Lys 27 (H3K27me3) promoters were specifically active only at day 2 (Figure 1F). These data demonstrated that hPSCs were efficiently induced into primitive streak cells at day 2.

Lateral Mesoderm Progenitors Require BMP4 Signaling and ROCK Inhibition

In phase 2 (D3-10, Figure 2A) of our platform, we aimed at differentiation into lateral mesoderm progenitors, which...
The presence of neurotrophin-4 (NT4) or ROCK inhibitor Y27632. Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05, **p < 0.01.

(E) ChIP-seq, using H3K4me3 and H3K27me3 antibodies, shows that the HAND1 promoter was fully active only at day 10.

(F) Quantification of surviving live cells by trypan blue staining, during mesoderm differentiation, in the presence of neurotrophin-4 (NT4) or ROCK inhibitor Y27632 (relative to NT4). Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05. **p < 0.01.

(G) Immunofluorescence staining shows that nearly 100% of cells at day 10 were positive for the lateral mesoderm TF: HAND1.

(H) Quantitative data showed that nearly 100% of cells at d10 were positive for HAND1 based on immunostaining against HAND1. Data are represented as mean ± SD, n = 3 independent experiments. The percentage of HAND1-positive cells from different views were counted for quantification.

(I) ChIP-seq, using H3K4me3 and H3K27me3 antibodies, shows that the HAND1 promoter was fully active only at day 10.

Figure 2. Lateral Mesoderm Differentiation Using BMP and ROCK Inhibition

(A) Schematic of three-phase protocol for differentiation of human iPSCs toward MSCs. Phase 1, the induction of primitive streak cells from human iPSCs; phase 2, differentiation into lateral mesoderm progenitors; phase 3, specification of hPSC-MSCs. The developmental stages were characterized by expression of phase-specific marker genes. A, activin A; C, CHIR99021; F, FGF; B, BMP4; R, Y27632; Fs, follistatin; P, PDGF; E, EGF; AA, ascorbic acid; PS, primitive streak.

(B) Titration of BMP4 (0–40 ng/mL) or the BMP antagonist Noggin, against lateral mesoderm differentiation, as determined by qRT-PCR for the mesoderm markers HAND1, FOXF1, and CD105, the endodermal TF GATA4, and the ectodermal TF SOX1. Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05, **p < 0.01.

(C) Titration of BMP4 or the BMP antagonist Noggin, against pluripotency markers, as determined by qRT-PCR for the pluripotency TFs OCT4, NANOG, and SOX2, at day 10 (relative to iPSCs). Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05, **p < 0.01.

(D) Phase contrast photomicrographs of BJ-iPSC- or MRC5-iPSC-derived primitive streak cells undergoing mesoderm differentiation, in the presence of neurotrophin-4 (NT4) or ROCK inhibitor Y27632.

(E) Quantification of surviving live cells by trypan blue staining, during mesoderm differentiation, in the presence of neurotrophin-4 (NT4) or ROCK inhibitor Y27632 (relative to NT4). Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05, **p < 0.01.

(F) qRT-PCR for the lateral plate mesoderm markers HAND1 and FOXF1, the endodermal TF GATA4, and the ectodermal TF SOX1. Data are represented as mean ± SD, n = 3 independent experiments. *p < 0.05.

(G) Immunofluorescence staining shows that nearly 100% of cells at day 10 were positive for the lateral mesoderm TF: HAND1.

(H) Quantitative data showed that nearly 100% of cells at d10 were positive for HAND1 based on immunostaining against HAND1. Data are represented as mean ± SD, n = 3 independent experiments. The percentage of HAND1-positive cells from different views were counted for quantification.

(I) ChIP-seq, using H3K4me3 and H3K27me3 antibodies, shows that the HAND1 promoter was fully active only at day 10.
A

Gene expression

CD73

**

IPS  FPT1  FPT1AA  FPE  FPEAA  FPT1E  FPT1EAA

B

Gene expression

CD105

**

IPS  FPT1  FPT1AA  FPE  FPEAA  FPT1E  FPT1EAA

C

Gene expression

CD44

**

IPS  FPT1  FPT1AA  FPE  FPEAA  FPT1E  FPT1EAA

D

Gene expression

SOX9

*

IPS  FPT1  FPT1AA  FPE  FPEAA  FPT1E  FPT1EAA

E

Gene expression

CD73

ns

IPS  hPS-MSC  BMSC

Gene expression

CD105

ns

IPS  hPS-MSC  BMSC

Gene expression

CD44

ns

IPS  hPS-MSC  BMSC

Gene expression

OCT4

ns

IPS  hPS-MSC  BMSC

Gene expression

SOX2

ns

IPS  hPS-MSC  BMSC

Gene expression

HAND1

*

IPS  hPS-MSC  BMSC

Gene expression

FOXA1

ns

IPS  hPS-MSC  BMSC

Gene expression

PAX6

ns

IPS  hPS-MSC  BMSC

F

IgG  D10  31.4%  D14  64.3%  D21  94.6%  BMSC  98%

G

CD73  OCT4  DAPI  Merge

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adipogenesis. Using different combinations and concentrations of the cytokines FGF, platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), and epidermal growth factor (EGF), whose receptors are highly expressed in primary bone marrow MSCs (BMSCs) (Liu et al., 2007; Ng et al., 2008), we assayed for the expression of classical MSC markers. Our results showed that FGF2, PDGF, and EGF, supplemented with ascorbate, were optimal for inducing the classical MSC markers: CD73 (ecto-5-nucleotidase), CD105 (endoglin), CD44 (hyaluronan receptor), and the osteochondrogenic master regulator SOX9 (Figures 3A–3D), and classical MSC morphology (Figure S5A).

During the optimization of factors to promote specification of skeletal MSCs from lateral mesoderm progenitors, we tested the effects of ascorbate—which we had found to promote primary BMSC self-renewal earlier (Figure S5B). In nearly every combination of cytokines, ascorbate supplementation could significantly increase the expression of MSC markers, indicating that ascorbate could promote MSC specification (Figures 3A–3D). qRT-PCR results also showed that the ascorbate-induced hPSC-MSCs, which manifested primary BMSC-like levels of CD73, CD105, and CD44 (Figure 3E), had extinguished all expression of the aforementioned pluripotency (OCT4, SOX2), mesoderm (MIXL1, HAND1), endoderm (FOXA1), and ectoderm (PAX6) Tsfs by day 21 (Figures 3E and S3).

FACS analysis confirmed that 94.6% of the MRC5-iPSC-derived cells were positive for the MSC marker CD73 by day 21, similar to primary BMSCs (Figure 3F). Similar results were obtained in other hPSCs: 97.2% for BJ-iPSC-derived cells and 95.2% for H1-human embryonic stem cell (hESC)-derived cells (Figure S6), showing that this defined protocol was highly reproducible. The CD73 qRT-PCR and FACS data were also further confirmed by immunofluorescence staining, which showed that nearly all of these cells were CD73+ MSCs (Figure 3G).

Characterization of hPSC-MSCs Relative to Primary BMSCs
To elucidate the molecular similarities and differences between hPSC-MSCs and primary BMSCs at the genomic level, the whole-genome transcriptome profiles of hPSC-MSCs and BMSCs were compared with each other. The correlation coefficient between the hPSC-MSCs’ transcriptome and the primary BMSCs’ transcriptome was very high, $r = 0.954$ (Figure 4A). Hierarchical clustering showed that hPSC-derived cells in the intermediate phases 1–3 showed a gradual transcriptomic and epigenomic transition from the hPSC state to the hPSC-MSC state, which was remarkably similar to the primary BMSC state (Figures 4B, 4C, and S7).

Gene set enrichment analysis of the genes differentially expressed between day 10 cells (phase 2) versus hPSC-MSCs (day 21, phase 3) and BMSCs, showed that the top signature upregulated during MSC specification is the epithelial-mesenchymal transition (EMT) signature (Figure 4D). This suggests that mesoderm derivatives only fully adopt a mesenchymal identity during MSC specification.

Detailed inspection of several well-known EMT markers, such as E-cadherin (CDH1) and TWIST1, confirmed this analysis (Figures 4E and 4F).

Multiplexed FACS profiling further showed that hPSC-MSCs displayed surface antigen profiles that were equivalent to primary BMSCs, being 100% negative for the blood lineage markers CD14, CD34, and CD45, while being nearly 100% positive for all the known BMSC surface markers CD29, CD44, CD49c, CD73, CD90, CD105, CD151, and CD166 (Figure 4G).

In addition, hPSC-MSCs also expressed high levels of Gremlin mRNA and protein (Figures 4H and 4I), which had been recently shown to be a functional marker of BMSCs and skeletal stem cells (Worthley et al., 2015). Compared with primary BMSCs, hPSC-MSCs manifested similar frequencies of colony-forming unit-fibroblasts when seeded at single-cell density, suggesting that they possess a self-renewal capacity equivalent to that of primary BMSCs under the same culture conditions (Figure 4J).

We also assessed the differentiation potential of hPSC-MSCs into the three mesenchymal lineages. After 2 weeks of adipogenesis, the adipogenic markers CEBPa, PPARγ, LPL, and aP2 were highly upregulated in adipocytes derived from hPSC-MSCs (Figure 4K). Correspondingly, hPSC-MSCs were induced into osteogenesis for 2 weeks, and the osteogenic markers osteocalcin (OC), osteopontin (OPN), and ALP were also highly upregulated (Figure 4K).

Figure 3. Ascorbate Promotes MSC Specification
(A–D) Extent of MSC specification, as determined by qRT-PCR for the classical MSC markers (A) CD73, (B) CD105, (C) CD44, and (D) SOX9, by using different combinations of FGF2 (F), PDGF (P), TGF-β1 (T1), EGF (E), and AA. Data are represented as mean ± SD, $n = 3$ independent experiments. *$p < 0.05$, **$p < 0.01$.

(E) qRT-PCR for the expression levels of MSC markers (CD73, CD105, CD44), pluripotency markers (OCT4, SOX2), the primitive streak markers MIXL1, the lateral mesoderm marker HAND1, the endoderm marker FOXA1, and the ectoderm marker PAX6, in hPSC-MSCs and primary BMSCs, relative to iPSCs. Data are represented as mean ± SD, $n = 3$ independent experiments. *$p < 0.05$, n.s., not significant.

(F) Flow cytometry for CD73 expression in MRC5-iPSC-derived MSCs, relative to BMSCs.

(G) Immunofluorescence staining shows that nearly 100% of cells at day 21 were positive for CD73, and none were positive for OCT4.
A

B

C

D

E

F

G

H

I

J

K

L

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The hPSC-MSCs were also induced into chondrogenesis under pellet culture conditions for 4 weeks. The chondrogenic markers COL2A1, COL10A1, AGC1, and SOX9, were also highly upregulated (Figure 4K). The hPSC-MSCs’ differentiated progeny were positive for oil red staining for lipid droplets during adipogenesis, positive for alizarin red staining for bone calcium phosphate deposits during osteogenesis, and positive for Alcian blue staining for cartilaginous sulfated proteoglycans and cartilaginous type II collagen during chondrogenesis, similar to primary BMSCs (Figure 4L). Thus, the hPSC-MSCs showed robust mesenchymal tri-lineage differentiation potential, similar to primary BMSCs. To validate the complete absence of pluripotent and tumorigenic (teratoma or sarcoma) stem cells, $2 \times 10^6$ hPSC-MSCs were subcutaneously transplanted into each of six immunodeficient mice. Zero tumors were observed after 4 months in all six mice.

hPSC-MSCs Require Ascorbate and Iron

In the presence of ascorbate, we found that newly derived hPSC-MSCs showed significantly improved chondrogenic potential, compared with hPSC-MSCs derived without ascorbate, according to qRT-PCR assays for chondrogenic markers (Figure 5A). Ascorbate supplementation significantly enhanced the expression of COL2A1 (α chain of type II collagen) and AGC1 (aggrecan, cartilage-specific proteoglycan core protein) mRNA, while repressing the expression of COL10A1 (α chain of type X collagen) mRNA, suggesting that ascorbate promotes hyaline cartilage rather than hypertrophic cartilage formation. This is important because hyaline cartilage is highly desirable for repairing joint articular cartilage in degenerative diseases, such as osteoarthritis. These mRNA results on hyaline cartilage were validated at the protein level by immunostaining for type II collagen and Alcian blue staining for sulfated proteoglycans under pellet culture conditions (Figure 5B). Ascorbate supplementation did not affect the expression of osteogenic genes (OC and COL1A1) in newly derived hPSC-MSCs that underwent osteogenesis. However, ascorbate supplementation decreased the expression of adipogenic genes (CEBPα and PPARγ) by about 2-fold in newly derived hPSC-MSCs that underwent adipogenesis (Figure 5B). Thus, our results showed that ascorbate specifically increased the chondrogenic potential of newly derived hPSC-MSCs after the specification of hPSC-MSCs.

Mechanistically, while ascorbate could serve as a cofactor to promote collagen protein synthesis (Murad et al., 1981), it could not explain the increase in collagen mRNA, and specifically the increase in COL2A1 mRNA and the decrease in COL10A1 mRNA. It also could not explain ascorbate’s induction of the MSC markers, CD73, CD105, and CD44, and the osteochondrogenic TF SOX9 (Figures 3A–3D).

Ascorbate metabolism is known to exert pleiotropic effects on cells. In fact, ascorbate could also serve as an antioxidant to scavenge reactive oxygen species (ROS), or help MSCs regenerate another cellular antioxidant, glutathione. Thus, to examine if redox regulation was the mechanistic basis of ascorbate’s effects on MSC specification, we applied the antioxidant N-acetyl-cysteine (NAC) and the ROS-producing hydrogen peroxide (H$_2$O$_2$) during the specification of hPSC-MSCs. Although we expected NAC to mimic ascorbate, and H$_2$O$_2$ to block ascorbate’s effects, we found the opposite to be true (Figure 5C). NAC robustly blocked the positive effects of ascorbate on MSC markers, whereas...
A

**COL2A1**

|    | MSC | FPEAA | FPE |
|----|-----|-------|-----|
| 700|     |       |     |
| 350|     |       |     |
| 0  |     |       |     |

**AGC1**

|    | MSC | FPEAA | FPE |
|----|-----|-------|-----|
| 40 |     |       |     |
| 20 |     |       |     |
| 0  |     |       |     |

**COL10A1**

|    | MSC | FPEAA | FPE |
|----|-----|-------|-----|
| 60 |     |       |     |
| 30 |     |       |     |
| 0  |     |       |     |

B

H&E

Alician blue stain

Type II Collagen

+AA

-AA

C

Gene expression

| Gene | PBS | PBS+AA | DFO | DFO+AA |
|------|-----|--------|-----|--------|
| CD44 | **| * | | |
| CD73 | **| * | | |
| CD105 | **| * | | |
| RUNX2 | ** | * | | |

D

K4me3

K9me3

K36me3

K27me1

K27me3

K4me1

K9me1

K30me1

H3

17 kDa

15 kDa

17 kDa

17 kDa

15 kDa

15 kDa

17 kDa

17 kDa

E

Gene expression

| Gene | CTRL | AA | KDM4B KD | KDM4B KD+AA |
|------|------|----|---------|-------------|
| CD44 | * | * | ** | ** |
| CD73 | * | * | ** | ** |
| CD105 | * | * | ** | ** |

F

Gene expression

| Gene | CTRL | AA | KDM4B OV | KDM4B OV+AA |
|------|------|----|---------|-------------|
| CD44 | * | * | ** | ** |
| CD73 | * | * | ** | ** |
| CD105 | * | * | ** | ** |

G

Cell number

| Passage Number | P27 | P28 | P29 | P30 | P31 | P32 | P33 |
|----------------|-----|-----|-----|-----|-----|-----|-----|
| MSC            |     |     |     |     |     |     |     |
| AA             |     |     |     |     |     |     |     |
| TF             |     |     |     |     |     |     |     |
| TF-AA          |     |     |     |     |     |     |     |

H

Gene expression

| Gene | MSC D0 | AA D0 | TF-AA D0 |
|------|--------|-------|----------|
| CD44 | * | * | * |
| CD73 | * | * | * |
| CD105 | ** | * | * |

I

Gene expression

| Gene | MSC | AA | TF | AA+TF |
|------|-----|----|----|-------|
| OC   |     |    |    |       |
| OPN  | ** | * | * |       |
| ALP  |    |    |    |       |

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H$_2$O$_2$ increased the MSC markers CD44, CD73, and CD105. Instead, these results suggested that some levels of ROS were in fact necessary for the specification of MSCs from lateral mesoderm progenitors, and that the antioxidant function of ascorbate could not have been the mechanistic reason for its promotion of MSC specification.

Another metabolic function of ascorbate is to serve as a cofactor for a family of enzymes known as the ascorbate/iron-dependent dioxygenases. If ascorbate was indeed promoting MSC specification via the ascorbate/iron-dependent dioxygenases, then it would imply that iron sequestration would block ascorbate’s positive effects on MSC markers. We found that some iron sequestration by low concentrations of desferrioxamine (DFO) did robustly abrogate the positive effects of ascorbate, without any toxicity to hPSC-MSCs on its own, indicating that DFO blocked MSC specification in an ascorbate-dependent manner (Figure 5C). Conversely, these data also implied that ascorbate promoted MSC specification in an iron-dependent manner.

One important subgroup of the ascorbate/iron-dependent dioxygenases is the JmjC histone demethylases that regulate the chromatin epigenetic state. To assess the effects of ascorbate on histone methylation during MSC specification, we performed a series of western blots for different histone H3 lysine residues’ methylation status. We observed that ascorbate robustly reduced H3K4me3, H3K9me3, H3K36me3, and H3K27me1, relative to the PBS control (Figure 5D). Most other H3 lysine methylation marks remained unchanged. These results suggest that ascorbate promoted MSC specification via a subgroup of JmjC histone demethylases. To test if ascorbate was acting on these H3 methyl-lysines via iron-dependent demethylases, we first added DFO to the cells during MSC specification, with and without ascorbate. Our results showed that only the specific H3 methyl-lysine marks reduced by ascorbate, were specifically increased by DFO (Figure 5D), indicating that the ascorbate-mediated H3 lysine demethylation was indeed iron-dependent.

To further test if ascorbate promoted the specification of hPSC-MSCs via JmjC ascorbate/iron-dependent histone demethylases, we knocked down and overexpressed the H3K9me3 demethylase KDM4B. KDM4B, also known as JMJD2B, is a JmjC domain-containing histone demethylase that is specifically involved in the demethylation of repressive H3K9 tri-methylation, which is one of the four H3 Lys residues affected by our ascorbate treatment of hPSC-MSCs. Our data showed that ascorbate supplementation alone increased the expression of KDM4B (Figures 5E and 5F), as well as other MSC markers. KDM4B knockdown with lentiviral short hairpin RNA (shRNA) decreased the expression of the MSC markers CD44, CD73, CD105, and RUNX2, both in the absence and presence of ascorbate, although ascorbate supplementation did partially rescue several MSC markers after KDM4B’s partial knockdown (Figure 5E). KDM4B lentiviral overexpression further increased the expression of MSC genes, both in the absence and presence of ascorbate (Figure 5F). These results suggest that KDM4B is both necessary and sufficient for hPSC-MSC specification, and that KDM4B lies downstream of ascorbate.

Conversely, when we supplemented the ascorbate-containing media with iron, by adding transferrin, we observed a large increase in the self-renewal capacity of hPSC-MSCs, which would otherwise undergo senescence and apoptosis over long-term culture (Figure 5G). Ascorbate-treated hPSC-MSCs still retained the capacity for colony formation...
and tri-lineage differentiation after long-term culture (Figure S9). Although either ascorbate or transferrin alone could promote hPSC-MSC proliferative potential, neither could increase hPSC-MSC proliferative potential as much as the combination of both ascorbate and transferrin (Figure 5G). Moreover, ascorbate/transferrin-treated hPSC-MSCs were also significantly higher in their expression of MSC markers than the vehicle control, especially the osteogenic TF RUNX2 (Figure 5H), suggesting improved osteochondrogenic differentiation potential after ascorbate/transferrin treatment. Indeed, we found that transferrin synergized with ascorbate to enhance the long-term osteogenic potential of hPSC-MSCs (Figure S1). Together with our chondrogenesis results (Figure 5B), our data support the idea that hPSC-MSCs require ascorbate and iron to maintain long-term self-renewal of MSC identity and long-term osteochondrogenic differentiation potential, both of which diminish with aging in MSCs.

**Repair of Cartilage Defects by hPSC-MSCs**

To assess the functionality of hPSC-MSCs in vivo, we sought to test their potency in repairing joint articular cartilage defects in vivo—potentially one of the most important applications of hPSC-MSCs. Primary BMSCs were considered as the gold standard to assess hPSC-MSCs. We orthotopically transplanted both hPSC-MSCs and primary BMSCs, which were subjected to chondrogenesis in vitro for 7 days under pellet conditions, into cartilage defects created in the joint articular surface of immunosuppressed rats (Liu et al., 2011). Six weeks after orthotopic transplantation, the defects were filled with engrafted tissue derived from the transplanted pellets. The engrafted tissue was positive for Alcian blue staining for sulfated proteoglycans and immunostaining for type II collagen, compared with the sham control (Figure 6A), showing that new cartilage similar to the adjacent host cartilage was being formed. Histological grading score showed that both hPSC-MSCs and primary BMSCs significantly improved cartilage repair compared with the sham control. There was no significant difference in the histological grading scores between hPSC-MSCs and primary BMSCs (Figure 6B). Immunostaining against the human-specific antigen lamin A/C confirmed that the engrafted human cells continued to be well-integrated into the adjacent host cartilage at 12 weeks (Figure 6F).

Taken together, our hPSC-MSCs fully resemble primary BMSCs epigenetically, transcriptionally and functionally, both in vitro and in vivo, as shown by their ability to repair joint articular cartilage defects. These data also suggest that ascorbate/transferrin-treated hPSC-MSCs could provide an alternative, patient-specific source of autologous cells for cartilage repair and regeneration in future.

**DISCUSSION**

By modeling the different phases of embryonic development that lead from hPSCs to skeletal MSCs, we have generated a platform to dissect human MSC specification. Our platform for generating skeletal MSCs from hPSCs represents a stepwise recapitulation of lateral mesoderm and appendicular skeleton development to derive MSCs with high efficiency. It is a chemically defined platform, freed of feeders to minimize the biological variability of the resultant MSCs, and a metabolically optimized platform to enhance MSCs’ long-term self-renewal and osteochondrogenic capacities.

In the process, we discovered that ROCK inhibition promotes lateral mesoderm progenitor proliferation, and that ascorbate promotes MSC specification. Moreover, we found that ascorbate promotes MSCs’ long-term self-renewal and osteochondrogenic potential in an iron-dependent fashion. This mechanism operated, in part, through ascorbate/iron-dependent dioxygenases, including a subgroup of JmjC histone demethylases.

Our histone methylation results suggested that at least four families of JmjC dioxygenases could be involved: the JMJD2 family of H3K9me3 demethylases, the JARID1 family of H3K4me3 demethylases, the JMJD3 family of H3K27me1 demethylases, and the JMJD2 family of H3K36me3 demethylases (Cloos et al., 2008). Our results further showed that KDM4B (JMJD2B) was necessary and sufficient to promote ascorbate-driven MSC specification, suggesting that ascorbate promoted the specification of hPSC-MSCs at least in part via the H3K9me3 demethylase KDM4B.

Given the importance of ascorbate/iron-dependent dioxygenases in cell fate decisions, the broad implications of optimizing the ascorbate/transferrin concentrations could
go beyond MSC specification in vitro. Already, it is well known that ascorbate is very useful in promoting iPSC re-programming (Chen et al., 2013; Chung et al., 2010; Wang et al., 2011). It is possible that tailoring the ascorbate/transferrin concentrations for particular cell types, beyond MSCs, could be useful for enhancing the specification and self-renewal of other adult stem cells in vitro. These findings also have important implications for our understanding of how nutritional supplementation with optimal ascorbate/iron concentrations could affect the self-renewal and maintenance of different stem cell populations in our body during aging in vivo. For example, it is thought that defects in adult MSC self-renewal, in part due to increasing H3K9me3 in aging bone, is responsible for osteoporosis.

Figure 6. hPSC-MSCs Can Fully Repair and Regenerate Articular Joint Cartilage

(A) After 6 weeks, H&E staining, type II collagen immunostaining, and Alcian blue staining of rat knee joint cartilage defects (dotted box) transplanted with hPSC-MSC- and primary BMSC-derived chondrogenic pellets, relative to the sham control. (B) Histological grading scores for rat knee joint cartilage defects after 6 weeks of transplantation with hPSC-MSC- and primary BMSC-derived chondrogenic pellets, relative to the sham control (**p < 0.01).

(C) Immunostaining for human-specific lamin A/C showed positive human chondrocytes integrated into the rat knee joint cartilage after transplantation with hPSC-MSCs and primary BMSCs, but not in the sham control after 6 weeks of transplantation.

(D) After 12 weeks, H&E staining, type II collagen immunostaining, and Alcian blue staining of rat knee joint cartilage defects (dotted box) transplanted with hPSC-MSC- and primary BMSC-derived chondrogenic pellets, relative to the sham control.

(E) Histological grading scores for rat knee joint cartilage defects after 12 weeks of transplantation with hPSC-MSC- and primary BMSC-derived chondrogenic pellets, relative to the sham control (**p < 0.01).

(F) Immunostaining for human-specific lamin A/C showed positive human chondrocytes integrated into the rat knee joint cartilage after transplantation with hPSC-MSCs and primary BMSCs, but not in the sham control after 12 weeks of transplantation.
during aging (Ye et al., 2012). Our results suggest that supplementing the correct ascorbate/iron concentrations for JmjC demethylases in MSCs in the human body might ameliorate the effects of aging on bone and cartilage diseases in future. Because the enzyme for ascorbate synthesis, L-gulonolactone oxidase, was only recently lost in primate evolution, it is possible that optimal ascorbate supplementation could be especially pertinent for somatic stem cells and their aging in human physiology.

**EXPERIMENTAL PROCEDURES**

**Generation of iPSCs**

Human iPSCs were generated by infecting fibroblasts of MRC5 or BJ cells with OCT4, SOX2, KLF4, or c-MYC retroviruses, as reported previously (Takahashi et al., 2007). GP2 cell were transduced with the four plasmids and the retrovirus was harvested 48 h after transduction. Fibroblasts were infected with harvested retrovirus. Five days after infection, infected MRC5 or BJ cells were plated onto mouse embryonic fibroblast feeder cells treated with mitomycin C and cultured in human ES medium to generate iPSCs. The generated iPSCs and the commercial hESC line H1 were used to define the differentiation protocol.

**Differentiation of hPSCs into MSCs**

Feeder-free human iPSCs were maintained in mTeSR (STEMCELL Technologies) before differentiation. To differentiate hPSCs into MSCs, hPSCs were digested with 1 mg/mL collagenase IV for 5–10 min at 37°C into small clumps and placed on a fibronectin-coated surface in medium containing activin A (25 ng/mL) and CHIR99021 (3 μM) for 1 day in a basal medium (DMEM: F12, 1% ITS, 2% B27, 2 mM L-glutamine, 90 μM β-mercaptoethanol), followed by activin A (25 ng/mL), CHIR99021 (3 μM), and FGF2 (20 ng/mL) for a further 24 h to differentiate toward primitive streak cells. Mesoderm differentiation was then induced with FGF2 (20 ng/mL), BMP4 (40 ng/mL), ROCK inhibitor Y27632 (5 μM), and follistatin (100 ng/mL) for 8 days. MSCs were then specified and matured with FGF2 (50 ng/mL), PDGF (50 ng/mL), EGF (100 ng/mL), and ascorbic acid (500 μg/mL) (Table S1) for another 11 days. The hPSC-MSCs were passage 1:3 with accutase and harvested with 0.25% trypsin/EDTA, washed and resuspended in FACS buffer for analysis. Cells were stained with PE-conjugated nonspecific immunoglobulin G to assess background fluorescence (Table S3).

**Osteogenic, Chondrogenic, and Adipogenic Differentiation Assays**

hPSC-MSCs were cultured under subconfluent conditions to prevent spontaneous differentiation. MSCs were induced to differentiate toward adipocytes in adipogenic medium and osteoblasts in osteogenic medium for 14 days, as described previously (Liu et al., 2007). A pellet culture system, as described previously (Liu et al., 2007), was used for chondrocyte differentiation for 28 days. Cells in growth medium were used as control. Adipogenic medium contained 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 1% antibiotic/antimycotic. Osteogenic medium contained 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 1% antibiotic/antimycotic. Chondrogenic medium contained 10 ng/mL TGF-β3, 0.1 μM dexamethasone, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL proline, 100 μg/mL pyruvate, and 50 ng/mL ITS+ Premix (Becton Dickinson; 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenious acid, 1.25 mg/mL BSA, and 5.35 mg/mL linoleic acid). The supplements were purchased from Sigma, unless otherwise stated. The medium was then changed every 3–5 days. Differentiation of MSCs was evaluated by qRT-PCR and lineage-specific staining: oil red O staining for lipid droplets in adipogenesis, alizarin red S staining for calcium deposits in osteogenesis, type II collagen immunostaining for the major collagen of cartilage, and Alcian blue staining for cartilage proteoglycans in chondrogenesis.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using an ABI PRISM 7900HT sequence Detection System (Applied Biosystems). In brief, 0.5 μg of total RNA was converted to cDNA using a high-capacity cDNA archive kit in 30 μL and then diluted to 500 μL. Diluted cDNA was used for qRT-PCR in a 384-well high-throughput format. For each individual qRT-PCR reaction per well of 10 μL, 5 μL of 2X SYBR Green Master Mix (Applied Biosystems) was used and combined with 0.5 μL of a combined forward and reverse primer mix (at 10 μM of forward + reverse primers in the combined primer mix). The expression of experimental genes was internally normalized to the expression of a human housekeeping gene (GAPDH) for that same cDNA sample.
Microarray Analysis

Total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Chatsworth, CA) per the manufacturer’s protocol. In brief, 0.3 μg total RNA was used to synthesize cRNA (Illumina TotalPrep RNA Amplification Kit, Ambion). The cRNA was then hybridized to Illumina Human HT-12 microarrays according to the manufacturers’ instructions. The data were analyzed using software GeneSpring v.12.5. All microarray data are available from the GEO (http://www.ncbi.nlm.nih.gov/geo) under accession code GSE140450.

ChIP-Seq

For ChIP-seq, samples were fixed with formaldehyde, lysed, sonicated, and precleared. The chromatin was probed overnight using H3K4me3 and H3K27me3 antibodies (Abcam) conjugated to Protein G Dynabeads (Invitrogen). Subsequently, chromatin was precipitated, rigorously washed, and de-crosslinked overnight at 65°C. Ten nanograms of chromatin were used to generate libraries (TruSeq Kit, Illumina) for HiSeq 2000 Sequencing (Illumina, 72 bp single-end reads). Reads were aligned to hg19.

Tumorigenicity Assay

To validate the loss of pluripotency in hPSC-MSCs, hPSC-MSCs were harvested by trypsinization and washed twice with PBS, viable cell number was determined by trypan blue exclusion. hPSC-MSCs (2 × 10⁶) suspended in Matrigel were subcutaneously transplanted into the flanks of 6-week-old immunodeficient nude mice. This assay was performed on six mice. The mice were kept in pathogen-free conditions and observed for 4 months to monitor tumor formation. All experiments involving animals were conducted under approved protocols granted by the A*STAR Institutional Animal Care and Use Committee.

Loss or Gain of Function of KDM4B

KDM4B was knocked down or overexpressed by lentiviral shRNA or cDNA, respectively, for stable expression. Lentiviral plasmids of KDM4B (Origene) were cotransfected with packaging vectors into 293FT cells. Supernatants were harvested after 48 h. Human lateral mesoderm progenitor cells at phase 2 of the differentiation protocol were infected with the lentiviral supernatants, containing 8 μg/mL Polybrene, to achieve stable knockdown or overexpression. The effects of knockdown or overexpression on the specification of hPSC-MSCs were examined at day 21 of differentiation.

Transplantation of hPSC-MSCs into Rats for Cartilage Repair Assay

hPSC-MSCs were transplanted into cartilage defects of rats as described previously (Liu et al., 2011). BMSCs were used as the gold standard control to assess hPSC-MSCs. BMSCs were used as positive controls in this study. In brief, male Sprague-Dawley rats (500 g) were anesthetized using an intraperitoneal injection of a mixture of ketamine (10 mg/100 g) and xylazine (1 mg/100 g). An anterior midline incision was made through the skin of the knee. The knee joints were opened via a medial parapatellar approach and the patella was everted. An osseochondral defect (1.5 mm in diameter and 1.5 mm in depth) was created in the patellar groove of the distal femur. Pellets from hPSC-MSCs or BMSCs were transplanted into cartilage defects, with the defects without transplanted cells serving as the control. The pellets from 3 × 10⁵ hPSC-MSCs or BMSCs were preinduced into chondrocyte differentiation for 1 week before transplantation as described previously (Liu et al., 2011). The recipient animals received daily subcutaneous injections of cyclosporine (14 mg/kg, Novartis Pharma AG, Basel, Switzerland) immediately after surgery.

At 6 and 12 weeks after surgery, rats from each group were sacrificed. The distal femurs with the cartilage defects were harvested and fixed in 10% buffered formalin. The tissues were decalcified and cut into 5-μm sections. Staining was performed with H&E, type II collagen immunostaining, and Alcian blue staining for sulfated proteoglycans in the cartilage matrix. Each sample was graded by an independent histopathologist according to the histological scale as described previously (Wakitani et al., 1994). The scale consists of five categories: cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration of donor with host cartilage. The scores range from 0 (normal articular cartilage) to 14 (no cartilaginous tissue), at least 10 defects from each group were assessed.

Statistical Analysis

Comparisons of histological scores for cartilage repair were performed using the Mann-Whitney U test for nonparametric analyses. Otherwise, statistical analyses were performed using Student’s unpaired two-tailed t tests. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.01.002.

AUTHOR CONTRIBUTIONS

E.D.Y., and D.V. performed the experiments. T.M.L., V.K., Y.H.L., S.M.C., B.T.T., and J.H.H., analyzed the data. B.L., N.S.-C., E.H.L., B.L., N.S.-C., S.M.C., B.T.T., and J.H.H., wrote the paper.

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