Elevated Osteogenic Potency by Defined Transcription Factors

Reprogramming of Dermal Fibroblasts into Osteo-Chondrogenic Cells with

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SUMMARY

Recent studies using defined transcription factors to convert skin fibroblasts into chondrocytes have raised the question of whether osteo-chondroprogenitors expressing SOX9 and RUNX2 could also be generated during the course of the reprogramming process. Here, we demonstrated that doxycycline-inducible expression of reprogramming factors (KLF4 [K] and c-MYC [M]) for 6 days were sufficient to convert murine fibroblasts into SOX9+/RUNX2+ cellular aggregates and together with SOX9 (S) promoted the conversion efficiency when cultured in a defined stem cell medium, mTeSR. KMS-reprogrammed cells possess gene expression profiles akin to those of native osteo-chondroprogenitors with elevated osteogenic properties and can differentiate into osteoblasts and chondrocytes in vitro, but form bone tissue upon transplantation under the skin and in the fracture site of mouse tibia. Altogether, we provide a reprogramming strategy to enable efficient derivation of osteo-chondrogenic cells that may hold promise for cell replacement therapy not limited to cartilage but also for bone tissues.

INTRODUCTION

The formation of skeletal tissues during embryonic development not only serves as a structural framework in our body to fulfill supportive, mechanical, and protective roles but also contributes to the homeostatic processes in the adult. The skeletal tissue is mainly derived from mesoderm with some elements originating from ectoderm. The formation of this is initiated when mesenchymal skeletal progenitor cells, also known as osteo-chondroprogenitors, undergo condensation and give rise to either chondrocytes to form the cartilage or osteoblasts to form the bone. The transcription regulators SOX9, a member of the Sox family, and RUNX2, a member of the Runt family, are co-expressed in osteo-chondroprogenitors, with the former being essential for its specification and chondrogenic lineage determination and the latter required for osteoblast cell-fate determination and early differentiation (Akiyama et al., 2005; Bi et al., 1999; Komori et al., 1997; Otto et al., 1997). In addition, several signaling molecules, notably transforming growth factor β1 (TGF-β1), bone morphogenetic proteins, fibroblast growth factors (FGFs), and WNTs are also crucial for establishing and directing differentiation of skeletal progenitor cells (Liu et al., 2008; Qi et al., 2014; Song et al., 2009). The ability to translate findings from these developmental studies to in vitro differentiation into chondrogenic and osteogenic lineages from pluripotent or multipotent embryonic stem cells (ESCs) has opened the door for the development of cell-based therapies for the treatment of a broad range of skeletal diseases (Hwang et al., 2008; Pelletari et al., 2008; Wu et al., 2013).

Pluripotent ESCs and induced pluripotent stem cells (iPSCs) represent a potentially unlimited source of skeletal cells for therapeutic applications. Several studies have established defined culture conditions to direct differentiation of mouse/human ESCs and human iPSCs into chondrogenic lineage (Craft et al., 2013; Umeda et al., 2015; Yamashita et al., 2015). In addition, the iPSC reprogramming approach has also been adopted in generating chondrocyte without passing through a stem cell state by ectopic expression of defined lineage-specific transcription factors based on their master-regulatory roles in chondrogenesis. It has been well established that SOX9, SOX5, and SOX6 (the SOX trio) are master regulators of chondrogenesis. Previous studies showed that conditional ablation of Sox9 gene in limb bud osteo-chondrogenitors prior to the onset of chondrogenic mesenchymal condensation resulted in a complete absence of cartilage and bone formation, whereas ablation of SOX9 function after mesenchymal condensation led to the impairment of chondrocyte proliferation and differentiation, which was predominantly mediated by the absence of Sox5 and Sox6 expression (Akiyama et al., 2002; Smits et al., 2001). In addition, the SOX trio regulates genes coding for the extracellular matrix components by binding to their enhancers (Bell et al., 2013;
Figure 1. Transient Formation of SOX9-EGFP+/RUNX2+ Nodules during Induction of Chondrogenic Cells from Dermal Fibroblasts by KLF4, c-MYC, and SOX9

(A) Schematic showing a direct conversion of mouse dermal fibroblasts (MDFs) prepared from newborn Sox9-EGFP knockin (KI) reporter mice into chondrocytes by retroviral-mediated expression of KLF4, c-MYC, and SOX9 (KMS). Scale bar, 50 μm. Transduced fibroblasts were cultured in the standard medium (DMEM + 10% FBS) and monitored daily for the formation of SOX9-EGFP+/RUNX2+ cells up to 14 days (d). MDFs did not express EGFP prior to retroviral transduction.

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adeno viral medi ated expres sion of the SOX trio is suffi cient to transform mouse der mal fi bro blasts (MDFs) into chondrogenic cells expressing cartilage marker genes and secre ting extracellular matrix. How ever, the induced cells still expressed fi brobl ast marker gene, type I collagen, which could impair the integrity of matrix structure and function, precluding the use of SOX trio induced cells for cartilage repair (Ikeda et al., 2004). This issue was resolved by another study in which MDFs were converted into chondrocytes without type I collagen gene expression by retro viral medi ated expression of two iPSC-reprogram ming factors (c-MYC and KLF4) and a master regulator for chondrogenesis, SOX9 (Hiramatsu et al., 2011). In addition, this chondrogenic induction did not pass through a pluripotent state throughout the period of the reprogramming process (Outani et al., 2011). Subsequently, the same research group generated induced chondrogenic (iChon) cells expressing type II but not type I COLLAGEN from human dermal fi broblasts with the same factors (Outani et al., 2013). Both mouse and human iChon cells produced homogeneous cartilage like tissues upon grafting in nude mice (Hiramatsu et al., 2011; Outani et al., 2013). However, it is not clear from these studies whether bipotential osteo-chondroprogenitors are generated during the direct conversion of fi broblasts into chondrocytes. Thus, it is conceivable that the ability to generate osteo-chondroprogenitors by a reprogramming approach with defi ned factors may hold promise for cell replacement therapy not limited to cartilage but also for bone tissues.

In the present study, we took advantage of this lineage-re programming approach to examine the possibility of osteo-chondroprogenitor formation using Sox9-EGFP knockin (Kl) reporter mice. We identifi ed osteo-chondrogenic cells during the course of lineage reprogramming from skin fi bro blasts to chondrocytes with gene expression profi les and in vitro differentiation potency comparable with native osteo-chondroprogenitors in developing mouse limb bud. Transplantation of reprogrammed osteo-chondrogenic cells subcutaneously and into bone lesion site of immunodefi cient recipients resulted in bone formation. Together, these fi ndings demonstrate that by using a transcription factor driven reprogramming approach we can effi ciently generate osteo-chondrogenic cells that readily form bone in vivo.

RESULTS

Transient Formation of SOX9-EGFP/RUNX2-Expressing Cells during Chondrogenic Induction from Skin Fibroblasts by KLF4, c-MYC, and SOX9
To determine whether cells expressing SOX9 and RUNX2 (SOX9*RUNX2*), which marks osteo-chondroprogenitors, could be formed during the course of chondrogenic induction by KLF4, c-MYC, and SOX9 (hereafter abbreviated as KMS), we performed retro viral medi ated expression of these factors in 2 × 10^4 MDFs prepared from new born Sox9-EGFP knockin (Kl) mice, which enabled us to monitor daily the expression of EGFP driven by cis-acting elements of Sox9 over 14 days of culture in conventional medium (DMEM + 10% fetal bovine serum [FBS]), a time when cells acquire chondrogenic features based on previous studies (Figure 1A; Hiramatsu et al., 2011). Thus, EGFP expression in transformed cells is considered to be an indicator of activation of Sox9 transcription followed by RUNX2 immuno- fluorescence to determine co-expression of SOX9-EGFP and RUNX2. We found that transformed fi broblasts began to form aggregates and express EGFP as early as day 9 (d9) post transduction. By d10, initiation of RUNX2 expression was detectable in EGFP⁺ aggregates with distinct nodular appearance, became signifi cant on d12 and d13, and diminished or were barely observed on d14, while EGFP was maintained from d9 to d14 (Figure 1B). By d14, cells in the aggregates exhibited typical polygonal-like morphology of chondrocyte with intense Alcian blue staining compared with MDFs in spindle shape, indicating production of proteoglycan in transformed cells (Figures 1B and 1C). Quantifi cation analyses showed that the number of nodules positive for SOX9-EGFP and RUNX2 (EGFP⁺/RUNX2⁺) increased from d10 (24.25% ± 0.829%) to d13 (63.03% ± 2.92%) and signifi cantly reduced on d14 (14.15% ± 3.31%). By contrast, the number of GFP⁻/RUNX2⁺ nodules formed were reduced from d11 (40.15% ± 0.59%) to d13 (9.54% ± 0.51%) but markedly increased on d14 (74.88% ± 1.63%). In addition, we also observed nodules negative for both EGFP and RUNX2 (GFP⁻/RUNX2⁻) from d9 (51.19% ± 1.12%) to d14 (23.20% ± 1.96%), but no nodules expressing RUNX2 alone were obtained throughout the period of analysis (Figure 1D). Taken together, these results suggest that transient expression of GFP⁺/RUNX2⁺ nodules were formed during KMS-mediated chondrogenic induction from skin fi broblasts.
Figure 2. Generation of SOX9-EGFP+/RUNX2+ Nodules by Dox-Inducible Lentiviral System

(A) Schematic diagram showing MDFs derived from Sox9-EGFP KI reporter mice were transduced with dox-inducible lentiviral KMS or KM vectors and treated with dox for 2, 4, 5, 6, 8, and 10 days to assess the formation of SOX9-EGFP+/RUNX2+ nodules.

(B) Panels show that no nodules were formed in MDFs transduced with KMS when treated with dox for 5 days, whereas nodules began to form on day 10 (d10), expressed GFP and RUNX2 from d11 to d13, and were positive for Alcian blue (inset in bottom right corner) on d14.

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Establishment of Culture Conditions to Promote the Formation of SOX9-EGFP+/RUNX2+ Cells

The inability of all nodules co-expressing SOX9-EGFP and RUNX2 to generate GFP+/RUNX2+ cells from d9 to d14, and the number of nodules co-expressing GFP and RUNX2 did not increase dramatically and even reduced on d13 compared with the condition without SNL feeder cells (Figures S1A and S1D). In addition, the number of GFP+/RUNX2+ nodules was markedly reduced under the hypoxic condition, which was shown to promote chondrogenesis via activation of Sox9 transcription (Figure S1B; Amarilio et al., 2007). These results suggest that SNL feeder or reduced oxygen content did not promote efficient generation of KMS-induced GFP+/RUNX2+ cells.

A defined ESC medium, mTeSR (Stem Cell Technologies), which mainly contains basic FGF (bFGF), TGF-β1, and LiCl, was previously found to promote the expansion of human mesenchymal stem cells that enrich for osteo-chondrogenic precursors expressing Sox9 and Runx2 genes (Hudson et al., 2011). We therefore tested whether mTeSR medium is able to increase the number of transformed GFP+/RUNX2+ nodules. We obtained a significant increase in the number of KMS-induced GFP+/RUNX2+ nodules by 18.44% ± 1.41% on d11 and 20.3% ± 2.92% on d13 compared with the conventional medium. In contrast, the number of GFP+/RUNX2+ nodules formed was markedly reduced on d14 (Figure 1E). Importantly, none of the nodules formed expressed either EGFP or RUNX2 only. These data suggest that mTeSR medium provided a better culture condition to promote a transient formation of KMS-induced GFP+/RUNX2+ cells and was therefore used for subsequent analyses.

On the other hand, it is also possible that KMS reprogrammed a small amount of tissue stem cells present in the dermal fibroblast culture into GFP+/RUNX2+ nodules (Sudo et al., 2007). To rule out this possibility, we transduced KMS into ESCs derived from Sox9-EGFP KI mice and cultured in mTeSR, and found that nodules transiently expressing GFP from d14 to d16 without RUNX2 co-expression were observed throughout the period of analysis (Figure S1C). The lack of sustained GFP expression could be due to either early silencing of transgene expression or lack of essential factors in ESCs to cooperate with the transgene for the maintenance of SOX9 expression. Nevertheless, the data suggest that transformed GFP+/RUNX2+ nodules were unlikely to be derived from resident stem cells in MDFs.

Generation of SOX9-EGFP+/RUNX2+ Nodules by Transient Expression of KMS or KM

Since KMS are oncogenes and their continued expression mediated by retroviral vectors in transformed cells could lead to tumor formation upon transplantation (Patel and Yang, 2010), we then examined whether transient expression of the transgene are sufficient to generate GFP+/RUNX2+ nodules. To this end, we transduced 2 × 10^4 MDFs with KMS using doxycycline (dox)-inducible lentiviral vectors and cultured with 1 μg/mL dox in mTeSR medium from d1 to d10 to monitor the formation of GFP+/RUNX2+ nodules until d14 (Figures 2A and S2A). While absence of dox treatment did not form GFP+/RUNX2+ nodules at all time points examined, we began to observe KMS-induced nodules formed on d10, expressing Sox9-EGFP/RUNX2 from d11 to d14 after withdrawal of dox from d7 onward, and they were intensely stained with Alcian blue on d14 (Figures 2B and S2A). No nodules expressing either SOX9-EGFP and/or RUNX2 were formed prior to 6 days of dox treatment (Figures 2B and S2A). Consistently, exogenous expression of Klf4, c-Myc, and Sox9 were markedly reduced by d8 following dox withdrawal on d7 (Figure 2D).

Importantly, the number of KMS-reprogrammed GFP+/RUNX2+ nodules formed between d11 and d14 were upon 6 days of dox treatment. Insets in top right corners show phase images of transduced cells and nodules cultured for 5 and 6 days of dox treatment, respectively.

(C) Quantification of the number of GFP+/RUNX2- and GFP+/RUNX2+ nodules formed from d9 to d14 in MDFs transduced with KMS following 6 days of dox treatment in culture.

(D) qPCR analysis of exogenous (ex) Klf4, c-Myc, and Sox9 expression in transduced cells at d2, d4, d6, and d8.

(E) MDFs transduced with KM were subjected to dox treatment for 5 and 6 days.

(F) Quantification of the number of GFP+/RUNX2- and GFP+/RUNX2+ nodules formed from d9 to d14 in MDFs transduced with KM following 6 days of dox treatment in culture.

(G) Comparison of the number of KMS- and KM-reprogrammed GFP+/RUNX2+ nodules formed from d10 to d14 following 6 days of dox treatment.

Three independent experiments are represented in (C), (D), (F), and (G). Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar in (B) and (E) represents 50 μm.
similar to those generated by retroviral transduction (compare Figures 2C and 1E). These results suggest that transient expression of KMS genes for at least 6 days is sufficient to generate SOX9-EGFP+/RUNX2+ nodules in kinetics and quantities similar to those generated by retroviral-mediated expression of the transgene.

We then investigated the minimum number of transcription factors required for the formation of induced GFP+/RUNX2+ nodules in mTeSR medium and found that MDFs transduced with KM genes were also capable of forming GFP+/RUNX2+ from d10 onward after 6 days of dox treatment, but an approximately 2-fold reduction of their numbers were obtained compared with KMS between d10 and d14 (Figures 2E and 2G). In contrast, the number of GFP+/RUNX2+ nodules formed was negligible upon transduction of KS genes (Figure S2B). In addition, fibroblasts transduced with either MS, K, or M genes did not form nodules but remained single cells largely scattered throughout the medium (Figures S2B and 2C). Some cells were occasionally positive for GFP and RUNX2. We did not observe fibroblasts expressing GFP and/or RUNX2 upon transduction of Sox9 gene throughout the period of analysis (Figure S2B). These results suggest that KLF4 and c-MYC are sufficient to convert fibroblasts into GFP+/RUNX2+ nodules, albeit at lower efficacy than that of KMS (Figure 2G).

Molecular Characterization of Reprogrammed SOX9-EGFP+/RUNX2+ Nodules

We then examined whether dox-inducible KMS-reprogrammed EGFP+/RUNX2+ nodules express genes characteristic of osteo-chondroprogenitors and/or other mesenchymal lineages by real-time qPCR. We isolated SOX9-EGFP+ osteo-chondroprogenitors by flow cytometry from embryonic day 10 (E10) mouse limb bud as a positive control and MDFs as a negative control. These sorted cells expressed GFP, SOX9, and RUNX2 in mTeSR medium, confirming their osteo-chondroprogenitor identity (Figure 3A). We then examined transcript levels of osteo-chondrogenic and chondrogenic lineage markers in GFP+/RUNX2+ nodules generated by dox-inducible KMS or KM between d11 and d14 because none of the markers examined were detectable prior to d11 (data not shown). We found that the levels of Sox9, Runx2, Col2a1, Sox5, and Sox6 transcripts gradually increased and peaked at d13, similar to their amount detected in sorted GFP+ limb bud cells (Figures 3B, 3C, and 4A–4C). By d14, most of the transcripts were maintained in KMS-reprogrammed nodules (Figures 3B and 4A–4C) except that Runx2 expression was low (Figure 3C), consistent with its downregulation of protein level (Figure 2B). In contrast, expression of these genes was relatively low...
Figure 4. KMS-Reprogrammed Nodules Express Markers of Osteo-Chondroprogenitor and Chondrogenic Lineages
(A–M) qPCR analysis for the indicated transcript levels in KMS-reprogrammed nodules from day 11 (d11) to d14. Individual mRNA expression levels were normalized to Gapdh with fold change relative to MDFs. GFP+ limb bud cells, chondrocytes, osteoblasts, and P10
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in KM-reprogrammed nodules (Figures S3A–S3E). Intriguingly, the cell-surface marker genes tetraspanin CD9 and ectonucleotidase CD73, previously shown to enrich osteo-chondroprogenitor population with elevated osteogenic properties from bone marrow cells, exhibited higher levels of transcripts in KMS-reprogrammed nodules than that of sorted GFP+ cells and KM-reprogrammed nodules (Figures 4D, 4E, S3F, and S3G). In addition, both KMS- and KM-reprogrammed nodules did not express or expressed at low levels of markers for hypertrophic chondrocyte Col10 and osteoblast lineage, such as osteopontin, osteocalcin, and osterix, at all time points examined (Figures 4F–4I and S3H–S3K). In contrast, Col1α1, which is expressed in MDFs and osteoblasts, was gradually reduced from d1 to d6 in both KMS- and KM-reprogrammed cells and remained at low level of expression from d7 to d14, similar to that of sorted GFP+ cells, indicating that Cola1 expression was silenced in MDFs during the course of reprogramming (Figures 4J and S3L). Since Sox9 and Runx2 genes are also expressed in the prehypertrophic chondrocytes, we examined the expression of genes characteristic of prehypertrophic chondrocytes, Ihh and Ppr. While growth plate from the tibial epiphyseal cartilage of neonatal mice expressed high levels of Ihh and Ppr transcripts, neither KMS nor KM activated their levels of expression, ruling out the possibility that SOX9-EGFP+ /RUNX2+ are prehypertrophic chondrocytes (Figures 4K, 4L, S3M, and S3N). Moreover, we also detected an elevated level of Gremlin 1 expression, which defines a distinct population of osteo-chondroreticular stem cells in the bone marrow (Wang et al., 2015), in KMS-reprogrammed nodules at d13 only, whereas its expression was low in KM nodules (Figures 4M and S3O), suggesting that GFP+/RUNX2+ nodules may possess skeletal stem cell features. Consistent with a previous report (Outani et al., 2013), nodules expressing dox-inducible KMS or KM were not pluripotent, as indicated by significantly reduced expression of Oct4 and Sox2 at all times examined compared with that of ESCs (Figures S3P and S3Q). On the other hand, examination of other mesenchymal lineage markers showed that KMS- and KM-reprogrammed nodules exhibited a low level of expression of the adipocyte marker, PPAR-γ1 (peroxisome proliferator-activated receptor γ1), which was highly expressed in adipocytes isolated from epidemal fat pads of adult mice as a positive control and undetectable in sorted SOX9-EGFP+ cells (Figure S3R). Lastly, we examined genes characteristic for mesenchymal stem cells (MSCs) in reprogrammed GFP+/RUNX2+ nodules and detected low levels of Mmp3 expression and a negligible amount of Igf2 transcripts in both treatments, compared with a significant amount of their transcripts present in mouse bone marrow-derived MSCs, indicating that reprogrammed GFP+/RUNX2+ nodules did not harbor molecular features of MSCs (Figures S3S and S3T). Altogether, these results suggest that reprogrammed nodules derived from dox-inducible KMS exhibited gene expression profiles closely associated with, if not identical to, native osteo-chondroprogenitors and may also contain a subpopulation with osteogenic potential (Figure 4N).

Differentiation Potential of Reprogrammed SOX9-EGFP+/RUNX2+ Nodules
To determine whether KMS- and KM-reprogrammed GFP+/RUNX2+ nodules are able to differentiate into mesenchymal lineages including chondrocytes, osteoblasts, and adipocytes, we subjected nodules obtained on d13, when the majority of them are GFP+/RUNX2+, to chondrogenic, osteogenic, and adipogenic media to interrogate their differentiation potential. After 14 days of culture, both KMS- and KM-reprogrammed GFP+/RUNX2+ nodules were positive for alizarin red S (bone) and Alcian blue (cartilage) staining, demonstrating their ability to differentiate into osteoblasts and chondrocytes, respectively (Figure 5A). Further molecular characterization revealed that four independent reprogrammed cells and sorted GFP+ limb bud cells after treatment with osteogenic medium exhibited comparable levels of osteoblast marker expression with bone marrow-derived osteoblasts as positive control, indicating their osteoblast identity (Figure 5B). However, they were not able to differentiate into adipocytes as shown by the absence of oil red O staining (Figure 5A). By contrast, MDFs did not undergo differentiation into the tri-lineages whereas the opposite occurred for MSCs, as expected (Figure 5A). These data suggest that KMS- and KM-reprogrammed GFP+/RUNX2+ nodules harbor osteo-chondrogenic potential in vitro. To further evaluate their differentiation capacity in vivo, we injected 1 × 105 KMS-derived reprogrammed cells obtained on d13 subcutaneously into the dorsal flanks of nude mice. After 6 weeks of transplantation, immunofluorescence analysis revealed that donor-derived GFP+ cells gave rise to tissues expressing RUNX2, OSTERIX, and TYPE I COLLAGEN (COL1), whereas chondrocyte markers, SOX9, and TYPE II COLLAGEN (COL2) were not detected (Figure 5C). In addition, positive staining for Von Kossa was observed in the tissue, indicating tibial growth plate served as positive controls. Three independent experiments are represented. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

(N) Heatmap showing expression levels for the indicated transcripts in KMS-reprogrammed nodules from d11 to d14 and in GFP+ limb bud cells.
mineralization, and histological analysis further confirmed their bone identity (n = 8/10, Figure 5C). In contrast, KM-reprogrammed cells formed neither bone nor cartilage tissues in vivo. These data suggest that KMS-reprogrammed cells exhibited osteogenic differentiation capacity in vivo. To further substantiate this observation, we transplanted $10^4$ KMS-reprogrammed cells obtained on d13 to the fracture site of adult mouse tibia at the time of injury. After 6 days of transplantation, the gifted GFP+ cells differentiated into tissues expressing RUNX2 and COL1 but not SOX9 within the fracture site (Figure 5D). Histological analysis revealed that the grafted cells gave rise to tissue with trabecular bone-like morphologies (n = 9/10; Figure 5D). Importantly, we did not observe formation of tumor tissues in both transplantation experiments (Figures 5C and 5D). Together, these findings further indicate that KMS-reprogrammed GFP+/RUNX2+ nodules, though expressing markers of osteo-chondroprogenitor and chondrogenic lineages, possessed elevated osteogenic potency in vivo.

DISCUSSION

Direct lineage reprogramming from somatic cells orchestrated by overexpression of defined transcription factors has been successfully adopted in generating various tissue-specific cell types and their stem-like precursors including neurons, neural stem cells, cardiomyocytes, chondrocytes, hepatocytes, hepatic stem cells, and blood progenitor cells (Efè et al., 2011; Hiramatsu et al., 2011; Huang et al., 2011; Ieda et al., 2010; Outani et al., 2013; Pang et al., 2011; Qian et al., 2012; Szabo et al., 2010; Thier et al., 2012; Vierbuchen et al., 2010; Yu et al., 2013). Whether a similar approach could be used to generate osteo-chondroprogenitors expressing SOX9/RUNX2 has not been shown. Here we closely investigated the course of lineage reprogramming from dermal fibroblasts to chondrocytes by using Sox9-EGFP reporter mice and immunocytochemistry for RUNX2, revealing that chondrogenic induction passed through an intermediate state of osteo-chondroprogenitor-like cells with gene expression profiles akin to those of native osteo-chondroprogenitors in developing mouse limb bud and osteogenic potency in vivo.

Our studies also revealed that reduced oxygen content did not promote the formation of SOX9+/RUNX2+ nodules, in contrast to previous studies showing that hypoxia controls differentiation of prechondrogenic cells partly through regulation of Sox9 gene expression (Amarilio et al., 2007). The reasons for the discrepancy are not clear. One possibility could be the lack of hypoxic response in the transformed cells. By contrast, we found that mTeSR medium provided a better culture condition for enhancement of SOX9 expression (Figure 5D). Histological analysis revealed that the grafted cells gave rise to tissue with trabecular bone-like morphologies (n = 9/10; Figure 5D). Importantly, we did not observe formation of tumor tissues in both transplantation experiments (Figures 5C and 5D).

Figure 5. Differentiation Potency of Reprogrammed SOX9-EGFP+/RUNX2+ Nodules

(A) Alizarin red S, Alcian blue, and oil red O staining were performed on in vitro differentiated nodules transduced with KMS or KM. Bone marrow-derived mesenchymal stem cells (MSCs) served as a positive control and MDFs as a negative control.

(B) qPCR analysis for the levels of indicated transcripts in four independent KMS-reprogrammed nodules and osteoblasts derived from GFP+ limb bud cells after treatment with osteogenic differentiation medium. Individual mRNA expression levels were normalized to Gapdh with fold change relative to sorted GFP+ limb bud cells. Osteoblasts served as a positive control. Three independent experiments are represented. Data are expressed as means ± SEM. **p < 0.001.

(C) Immunofluorescence with antibodies against GFP, RUNX2, OSTERIX, COL1, COL2, and SOX9 (inset) on paraffin sections of tissues derived from KMS-reprogrammed cells. White dotted lines in the inset indicate tissue boundary. Representative Von Kossa and H&E staining of sections indicate formation of bone tissue.

(D) Phase-contrast image of transplanted KMS-reprogrammed nodule into the fracture site of mouse tibia (dotted box with arrow). Immunofluorescence with antibodies against GFP, RUNX2, COL1, and SOX9 on sections of mouse fracture tibia transplanted with KMS-reprogrammed cells. White dotted lines indicate tissue boundary. Representative H&E staining of serial section shows grafted tissue with trabecular bone-like morphologies.

Scale bars, 30 μm (A) and 100 μm (C and D).
reprogramming events even in the absence of continued transgene expression. It has been demonstrated that c-MYC and KLF4 are oncogenic factors, which induce epigenetic changes in gene expression to trigger transformation of fibroblasts into iPSCs when combined with OCT4 and SOX2 (Yamanaka and Takahashi, 2006). In our case, we speculate that KLF4 and c-MYC activated endogenous Sox9 and Runx2 gene expression during the reprogramming process to levels sufficient to give rise to osteo-chondrogenic lineages in vitro but not in vivo. They could also gradually silence expression of the fibroblastic marker Col1a1 from d1 to d6, which coincides with the duration of transgene expression required for chondrogenic induction, suggesting that complete silencing of Col1a1 expression by the transgene is required for the formation of osteo-chondroprogenitor-like cells. Addition of SOX9 first directs and promotes the efficiency of cell reprogramming into osteo-chondrogenic-like cells, before chondrogenic differentiation. This finding is consistent with the role of SOX9 in specifying osteo-chondroprogenitors and chondrogenic lineage by activating its downstream target genes (Col2a1, Sox5, and Sox6) during early limb bud development (Akiyama et al., 2005; Bi et al., 1999). In addition, the reprogrammed cells also expressed high levels of CD9 and CD73 expression, which are low in naive osteo-chondroprogenitors, and these surface markers have been used to enrich an osteo-chondroprogenitor population with augmented bone-forming capacity in vivo (Singh et al., 2015). Indeed, our transplantation studies demonstrated the ability of reprogrammed cells to differentiate into bone tissues subcutaneously and in the fracture site. In contrast, these cells could undergo chondrogenic and osteogenic differentiation, but not adipocyte differentiation, when cultured under differentiating conditions, indicating that they possess osteo-chondrogenic potential to a certain extent in vitro. However, we cannot rule out the possibility that these reprogrammed osteo-chondrogenic cells have “endochondral-like” potential that form bone via a cartilage intermediate in vivo. Collectively, KMS-reprogrammed osteo-chondrogenic cells differ from limb bud osteo-chondroprogenitors, with unique cell surface properties that confer their differentiation bias into osteo-chondrogenic lineage.

In addition to reprogramming fibroblasts into chondrocytes, a recent study also demonstrated a direct conversion of human placental cells to chondrocytes by a different set of transcription factors (Ishii et al., 2012). Whether osteo-chondrogenic cells could also be formed during this conversion remains to be determined. Nevertheless, we report here the use of defined factors to generate osteo-chondrogenic cells with higher bone-forming potency from skin fibroblasts. This study has paved the way for the generation of patient-specific osteo-chondrogenic cells as a possible new cell source for osteoblasts for the treatment of bone defects.

EXPERIMENTAL PROCEDURES

Mice and Cells
Mice were maintained in the Laboratory Animal Unit of the University of Hong Kong. All mouse studies were approved by the Committee on the Use of Live Animals in Teaching and Research and were carried out in accordance with institutional and international standards and regulations. MDFs were isolated from dermis of newborn Sox9-EGFP knockin (KI) mice. Skin tissues were manually dissociated and digested in 0.25% trypsin (Sigma) for 10–15 min at 37°C for 4 hr. After removing debris, cells were then plated onto a 24-well plate and incubated in conventional medium (DMEM [Gibco] plus 10% FBS [Biosera]).

Viral Infection
Retroviral vectors (pMX) carrying mouse KLF4 (ID: 13370) and c-Myc (ID: 13375) genes were purchased from Addgene deposited by S. Yamanaka (Center for iPS Cell Research and Application [CiRA], Kyoto University). pMX-Sox9 was generated by subcloning of mouse Sox9 full-length cDNA into a blunt-end pMX vector. Each retroviral DNA was transfected into Plat-E cells (a gift from A. Bradley, Sanger Institute) at 8 × 10⁶ cells per 10-cm dish by Lipofectamine 2000 (Invitrogen) and cultured with DMEM plus 10% FBS in the absence of antibiotics overnight at 37°C and 5% CO₂. The following day, cells were replaced with fresh medium and incubated for another day. The virus-containing medium was then collected and filtered through a 0.45-μm pore size cellulose acetate filter before mixing with 4 μg/mL Polybrene (Sigma). The medium in MDF culture dish was then replaced with 2 mL of Polybrene/virus-containing mixture in each well of a 24-well plate and incubated overnight at 37°C and 5% CO₂. The following day, the mixture was replaced with DMEM/10% FBS medium and monitored daily for GFP expression for up to 2 weeks.

Doxycycline-inducible lentiviral constructs pLe6d-Piglight-kcMYC, pLe6d-Piglight-kFLK4, and retroviral pMXs-gw/(rtTA2-M2) were gifts from N. Tsumaki (CiRA, Kyoto University). Lentiviral vectors were transfected into 293T cells at 6 × 10⁶ cells per 10-cm dish by Lipofectamine 2000 reagent (Invitrogen) and ViralPowerPackaging Mix (Invitrogen) and cultured in Opti-MEM Medium (Gibco) without serum in the absence of antibiotics overnight at 37°C and 5% CO₂. The following day, cells were replaced with fresh medium and incubated for another day. Virus-containing supernatants were harvested 48–72 hr post transfection, concentrated by PEG-it (System Bioscience), and stored at −80°C. MDFs from Sox9-EGFP KI mice were transduced with rtTA2-M2 retrovirus overnight prior to infection with inducible c-MYC, KLF4, and Sox9 lentiviruses for 48 hr and replaced with fresh virus medium every 12 hr. Afterward, MDFs were cultured with mTeSR without serum in the absence of antibiotics overnight at 37°C and 5% CO₂. The following day, dox was withdrawn from the transduced MDFs every 2 days and cultured until days 10–14 for immunofluorescence with GFP and Runx2 antibodies.
Flow Cytometry
Limb bud was dissected from both wild-type and Sox9-EGFP KI mouse embryos at E10 and digested in 0.25% trypsin at 37°C for 10 min. Cells were then centrifuged at 1,000 rpm for 5 min and resuspended in DMEM/10% FBS medium. Wild-type limb bud cells were used for calibration in FACSAria I (Becton Dickinson) at the Faculty Core Facility, Li Ka Shing Faculty of Medicine, the University of Hong Kong before proceeding to sort SOX9-EGFP+ cells. The sorted GFP+ cells were collected in FBS and plated at 1 × 10⁴ cells per well in a 24-well plate in DMEM/10% FBS at 37°C.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.04.018.

AUTHOR CONTRIBUTIONS
Y.W., M.H.S., D.C., K.S.E.C., and M.C. planned the experiments; Y.W., M.-H.W., and M.P.L.C. performed research and acquired data; H.A. generated Sox9-EGFP knockin mice; M.H.S., D.C., K.S.E.C., and M.C. supervised the project; Y.W. and M.C. analyzed data and wrote the manuscript.

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