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Article

Reprogramming of Dermal Fibroblasts into Osteo-Chondrogenic Cells with Elevated Osteogenic Potency by Defined Transcription Factors

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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; Pelttari 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.,
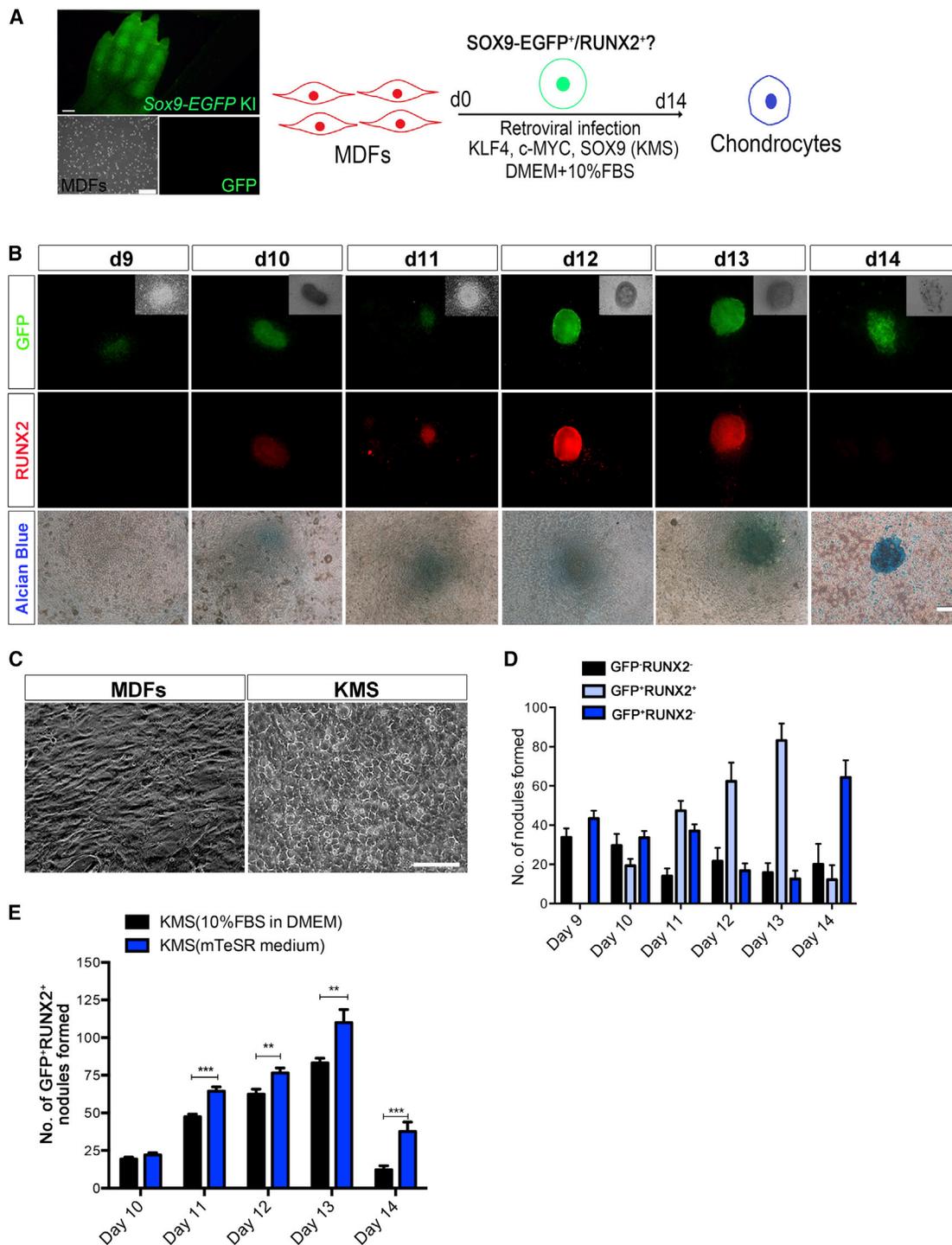
Figure 1. Transient Formation of SOX9-EGFP⁺/RUNX2⁺ Nodules during Induction of Chondrogenic Cells from Dermal Fibroblasts by KLF4, c-MYC, and SOX9

(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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1997; Bridgewater et al., 1998; Han and Lefebvre, 2008; Lefebvre et al., 1998; Nagy et al., 2011). Consistently, adenoviral-mediated expression of the SOX trio is sufficient to transform mouse dermal fibroblasts (MDFs) into chondrogenic cells expressing cartilage marker genes and secreting extracellular matrix. However, the induced cells still expressed fibroblast 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 retroviral-mediated expression of two iPSC-reprogramming 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 fibroblasts 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 fibroblasts into chondrocytes. Thus, it is conceivable that the ability to generate osteo-chondroprogenitors by a reprogramming approach with defined 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-reprogramming approach to examine the possibility of osteo-chondroprogenitor formation using *Sox9-EGFP* knockin (KI) reporter mice. We identified osteo-chondrogenic cells during the course of lineage reprogramming from skin fibroblasts to chondrocytes with gene expression profiles 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 immunodeficient recipients resulted in bone formation. Together, these findings demonstrate that by using a transcription factor-driven reprogramming approach we can efficiently 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 retroviral-mediated expression of these factors in 2×10^4 MDFs prepared from new born *Sox9-EGFP* knockin (KI) 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 fibroblasts 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 significant 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). Quantification analyses showed that the number of nodules positive for SOX9-EGFP and RUNX2 (GFP[+]/RUNX2^{+}) increased from d10 (24.25% ± 0.829%) to d13 (63.03% ± 2.92%) and significantly 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 fibroblasts.

(B) Insets show phase images of KMS-transduced MDFs with nodular appearance from d9 to d14. Immunofluorescence was performed on SOX9-EGFP[+] nodules with anti-GFP and anti-RUNX2. Induced nodules were stained with Alcian blue. Scale bar, 50 μm.
(C) Micrographs of MDFs and KMS-transduced cells on d14. Scale bar, 200 μm.
(D) Quantification of KMS-induced nodules formed upon cultured in the conventional medium. n = 3 independent experiments, mean ± SD.
(E) Quantification of KMS-induced GFP[+]/RUNX2^{+} nodules formed upon culture in conventional and mTeSR media. n = 3 independent cultures, mean ± SEM. **p < 0.01, ***p<0.001; Student's t test.
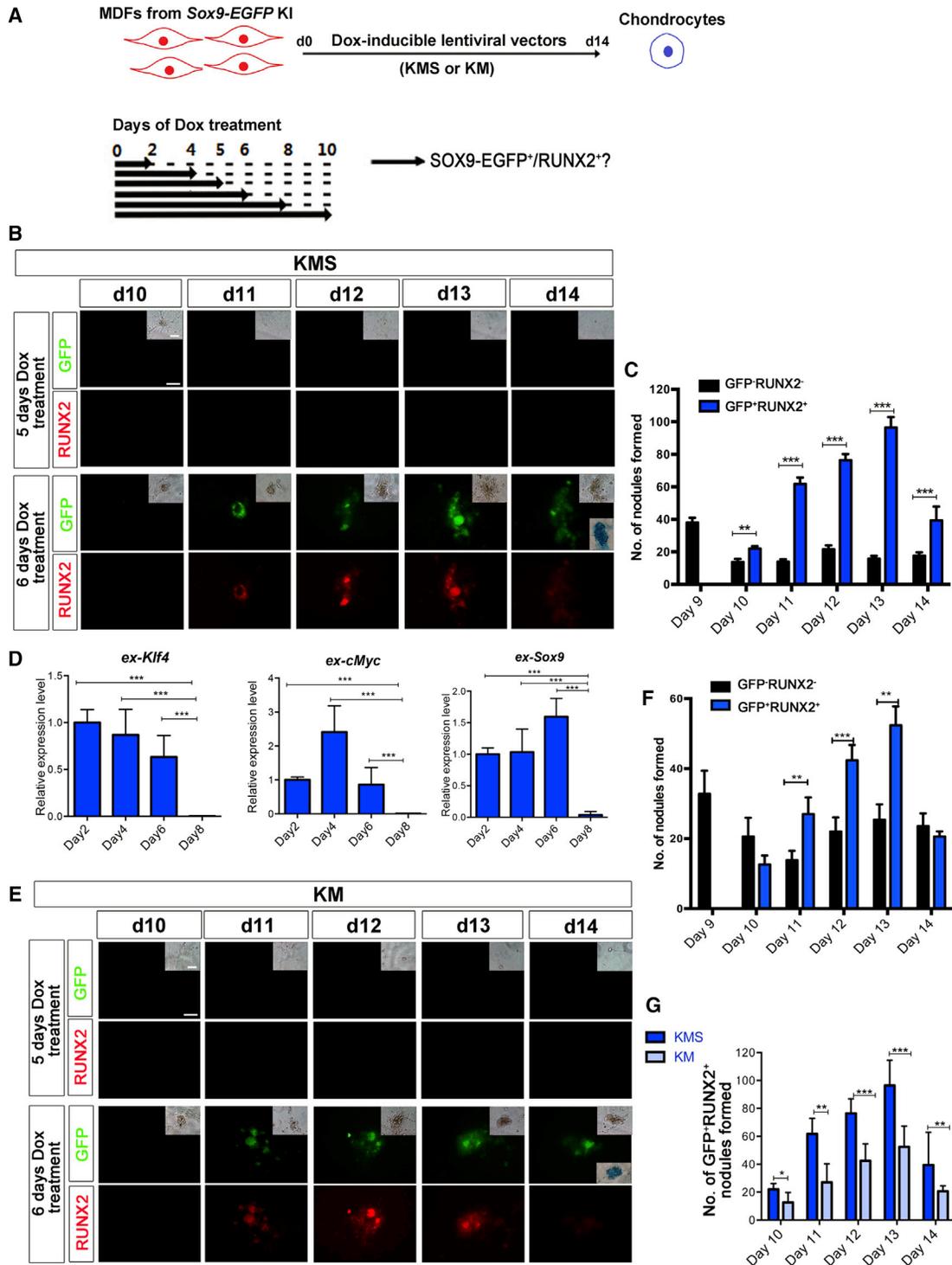
Figure 2. Generation of SOX9-EGFP⁺/RUNX2⁺ Nodules by Dox-Inducible Lentiviral System

(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 suggested a lack of essential components in culture medium to promote SOX9 and RUNX2 expression in transformed cells. To exploit an appropriate culture condition for the transduced cells, we first plated KMS-transduced cells onto SNL feeder cells, which are commonly used as a feeder layer for the propagation of mouse ESCs, and examined their ability to support the generation of GFP[+]/RUNX2^{+} cells. However, nodules expressing GFP alone were still observed 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 1D). In addition, the number of GFP[+]/RUNX2^{+} nodules was also 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.
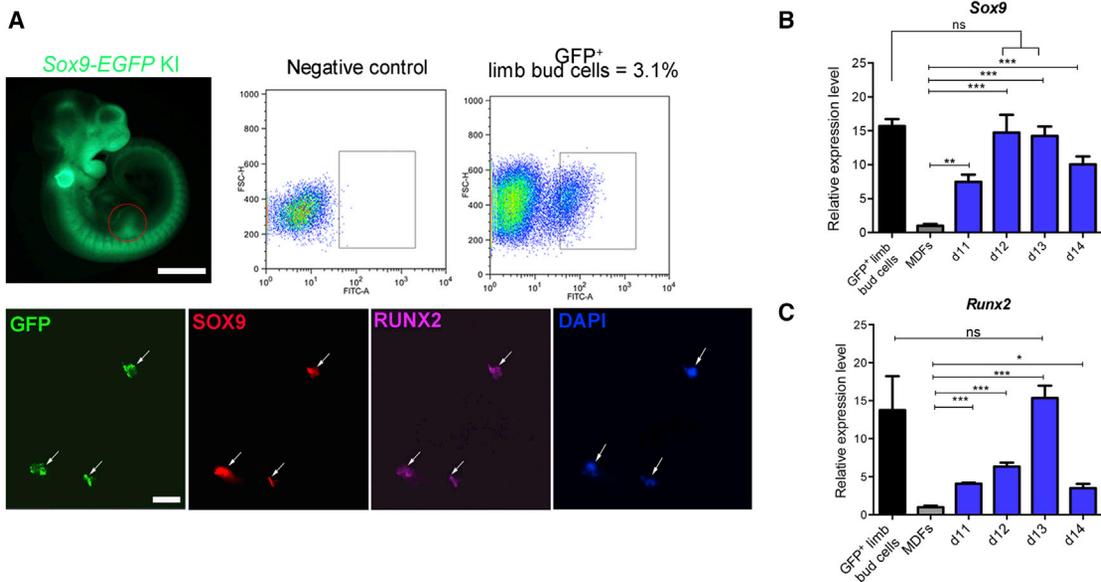
Figure 3. Expression of *Sox9* and *Runx2* in KMS-Reprogrammed Nodules

(A) (Top) Isolation of SOX9-EGFP⁺ cells from E10.5 mouse limb bud (red circle) by fluorescence-activated cell sorting as a positive control for the real-time qPCR analysis. Wild-type limb bud cells were used as negative control. Scale bar, 500 μm. (Bottom) Immunofluorescence of sorted GFP⁺ limb bud cells with antibodies against SOX9 and RUNX2 and cell nuclei were counterstained with DAPI (white arrows). Scale bar, 5 μm.

(B and C) qPCR analysis of *Sox9* (B) and *Runx2* (C) transcript levels in sorted GFP⁺ limb bud cells, MDFs, and KMS-reprogrammed nodules from day 11 (d11) to d14. Three independent experiments are represented. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

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
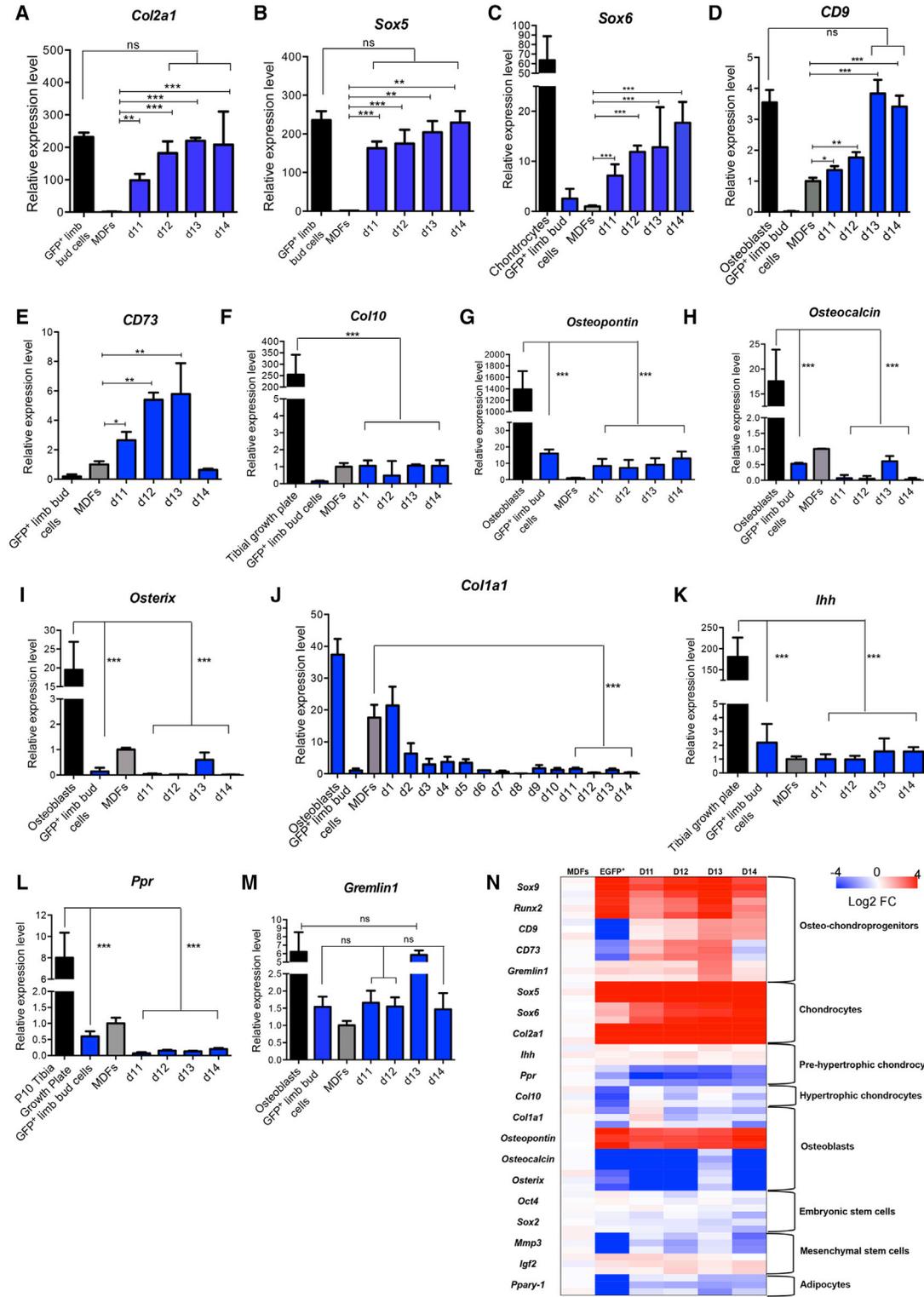
Figure 4. KMS-Reprogrammed Nodules Express Markers of Osteo-Chondroprogenitor and Chondrogenic Lineages

(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, *Col1a1*, 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 points 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 epidermal 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 1×10^5 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.
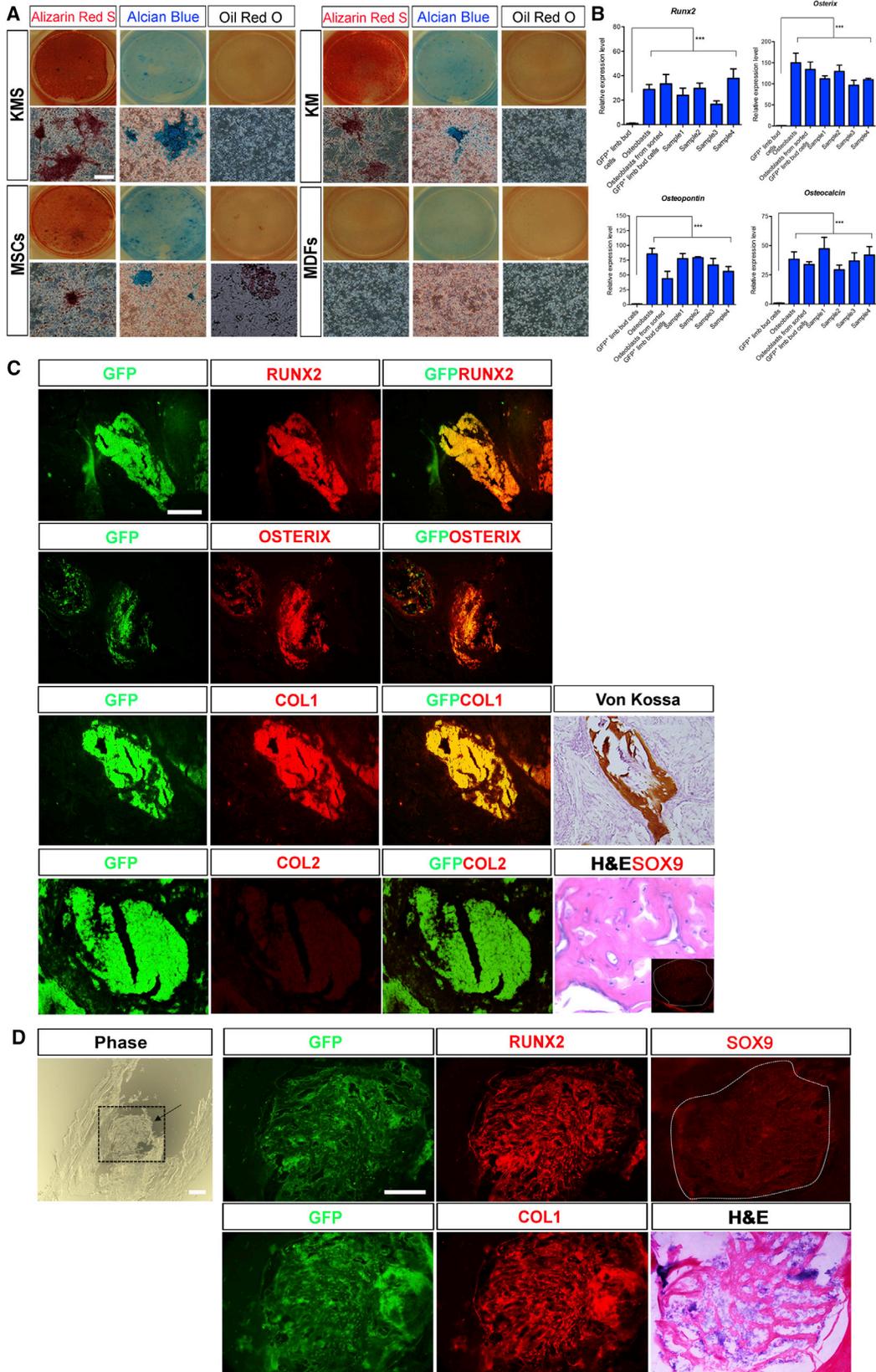
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mineralization, and histological analysis further confirmed their bone identity (n = 8/10, Figure 5C). In contrast, KM-reprogrammed cells formed neither bone nor cartilage tissues in vivo. These data suggest that KMS-reprogrammed cells exhibited osteogenic differentiation capacity in vivo. To further substantiate this observation, we transplanted 1×10^4 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 grafted 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 (Efe 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 chon-

drocytes 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 prechondrogeneic 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 the formation of SOX9-EGFP$^+$/RUNX2$^+$ nodules during chondrogenic reprogramming. A previous study showed that mTeSR mainly contains bFGF, TGF-β1, and LiCl, capable of supporting the preferential enrichment of multipotent human MSCs with osteo-chondrogenic potential (Hudson et al., 2011). TGF-β1 and FGF2 have been shown to be the inducers of *Sox9* gene expression and thus promote chondrogenesis in mesenchymal lineages, while LiCl is an activator of canonical Wnt signaling that was shown to promote osteogenesis by directly stimulating *Runx2* gene expression via its downstream effector, β-catenin (Gaur et al., 2005; Handorf and Li, 2011; Lorda-Diez et al., 2009). Altogether, our data suggest that the presence of these essential components in mTeSR medium could facilitate the induction of *Sox9* and *Runx2* gene expression in reprogrammed nodules.

Our results further revealed that transient expression of *KMS* genes for at least 6 days led to the subsequent formation of SOX9-EGFP$^+$/RUNX2$^+$ nodules between d11 to d13 in similar kinetics and quantities to those generated by retroviral-mediated transgene expression, suggesting that these factors are sufficient to promote and sustain

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-chondrogenitors, with unique cell surface properties that confer their differentiation bias into osteogenic 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 blunted pMX vector. Each retroviral DNA was transfected into Plat-E cells (a gift from A. Bradley, Sanger Institute) at 8×10^6 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% CO_2. 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% CO_2. 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-Ptight-hc-*MYC*, pLe6d-Ptight-hF(−)*SOX9*, pLe6d-Ptight-h*KLF4*, and retroviral pMXs-gw/(rtTA2-M2) were gifts from N. Tsumaki (CiRA, Kyoto University). Lentiviral vectors were transfected into 293T cells at 6×10^6 cells per 10-cm dish by Lipofectamine 2000 reagent (Invitrogen) and ViraPowerPackaging Mix (Invitrogen) and cultured in Opti-MEM Medium (Gibco) without serum in the absence of antibiotics overnight at 37°C and 5% CO_2. 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 in the absence or presence of 1 μg/mL dox. Medium was replaced every other 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 1×10^4 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.

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