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RAPID COMMUNICATION

SV40 large T antigen-induced immortalization reprograms mouse cardiomyocyte progenitors with mesenchymal stem cell characteristics and osteogenic potential

While progenitor cell-based cardiomyocyte regeneration holds great promise of repairing an injured heart, primary cardiomyogenic progenitors (CPs) have a limited life span in culture, hampering the use of CPs for *in vitro* and *in vivo* studies. We previously isolated primary CPs from mouse E15.5 fetal heart, and reversibly immortalized them with SV40 large T antigen (SV40 LTA), resulting in immortalized CPs (iCPs), which maintain long-term proliferation and express cardiomyogenic markers and retain differentiation potential under appropriate differentiation conditions.[1] The SV40 LTA plays essential roles in the simian virus 40 infection of permissive cells for the production of progeny virions, and in the infection of non-permissive cells, leading to malignant transformation or immortalization. While, as one of the most commonly-used immortalizing genes, SV40 LTA is thought to immortalize primary cells depending on its ability to complex with and hence inhibit p53 tumor suppressor,[2] the exact mechanism underlying SV40 LTA-mediated immortalization remains unknown. It was shown that induced pluripotent stem cells (iPSCs) could be established from somatic fibroblasts by simultaneous introduction of the four transcription factors OCT4, KLF4, SOX2, and C-MYC (also known as iPS factors or Yamanaka factors), and that reduced p53 expression facilitated reprogramming process.[3] We hypothesized that SV40LTA-mediated immortalization may at least partially resemble the reprogramming phenomenon. Using the most potent osteogenic factor BMP9,[4,5] we demonstrated that the immortalized cardiomyocytes exhibited mesenchymal stem cell (MSC) characteristics and osteogenic differentiation potential.

To investigate whether SV40 LTA could render the iCP cells MSC characteristics and whether the iCP cells would undergo osteogenic differentiation upon BMP9 stimulation, we chose two of the previously characterized iCP clones, namely iCP15 and iCP18 [1]. Since the iCP cells were reversibly immortalized with SV40 LTA flanked with LoxP sites (Fig. S1A), we first sought to determine if the immortalization phenotype of the iCP cells were dependent on SV40 LTA expression. We found that, when the iCP15 and iCP18 cells were effectively transduced with Ad-CRE or Ad-GFP control (Fig. S1B, a and b), the numbers of viable cells in the Ad-CRE infection groups markedly decreased (Fig. S1C, a), and quantitative analysis indicated that the reduced cell viability in the Ad-CRE groups was statistically significant (Fig. S1C, b). These results demonstrate that CRE-mediated removal of SV40 LTA diminishes the proliferative activity and survival of the iCP15 and iCP18 cells.

We next tested whether the iCPs exhibited MSC-like characteristics and were able to undergo osteogenic differentiation, an important lineage fate of MSCs. As we previously demonstrated that BMP9 is one of the most potent osteogenic factors,[4,5] we analyzed whether BMP9 would induce osteogenic differentiation of the iCP cells. When the iCP15 and iCP18 cells were infected with Ad-BMP9, the activities of early osteogenic marker alkaline phosphatase (ALP) assessed by histochemical staining were drastically upregulated by BMP9 at all three time points (Fig. 1A, panel a). Quantitative analysis revealed that the ALP activities significantly increased upon BMP9 stimulation in both iCP15 and iCP18 cells (Fig. 1A, panel b).

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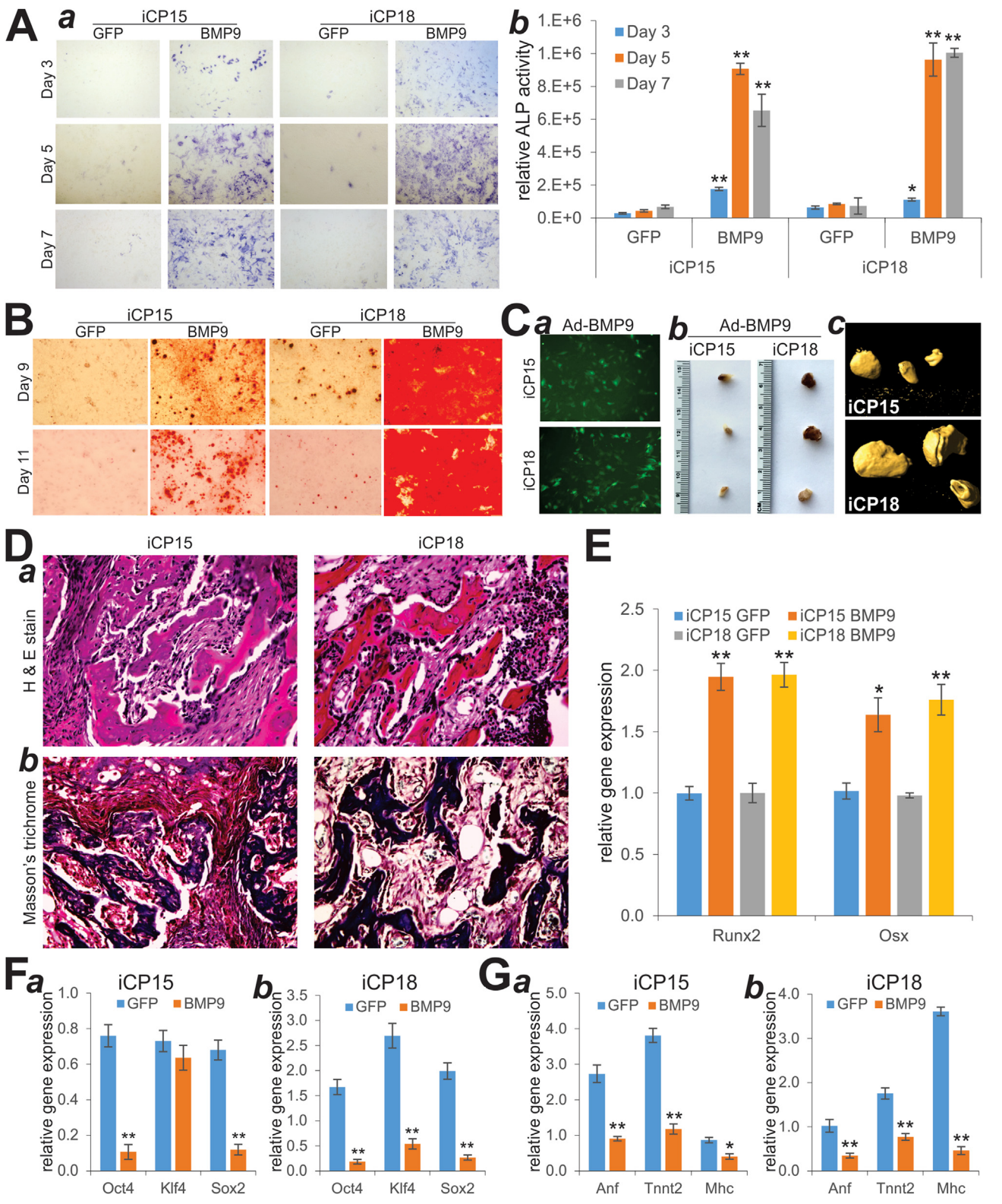

Figure 1 SV40 large T antigen renders mouse cardiomyocyte progenitors MSC characteristics and osteogenic potential. **(A)** The activities of BMP9-induced early osteogenic marker alkaline phosphatase (ALP) in iCP cells. Subconfluent iCP15 and iCP18 cells were infected with Ad-BMP9 or Ad-GFP. Qualitative histochemical staining assay of ALP activity was carried out at day 3, 5, and 7 after infection and observed under light microscope (×200). **(a).** Representative images are shown. **(b)** ALP activities were also quantitatively analyzed at the same time points. $^{*}P < 0.05$, $^{**}P < 0.01$, versus GPG groups at the same time points **(B)** BMP9-induced matrix mineralization in iCP cells. Subconfluent iCP15 and iCP18 cells were infected with Ad-BMP9 or Ad-GFP, and cultured in

Furthermore, both iCP15 and iCP18 cells were able to form robust matrix mineralization upon BMP9 stimulation (Fig. 1B). Interestingly, upon BMP9 stimulation the iCP18 cells seemingly exhibited higher osteogenic activity, compared with the iCP15 cells. Nonetheless, these *in vitro* results strongly indicate that both iCP15 and iCP18 cells were highly osteogenic upon BMP9 stimulation.

We further conducted animal experiments to determine whether the iCP cells were able to form ectopic bone *in vivo*. When iCP15 and iCP18 cells were effectively infected with Ad-BMP9 or Ad-GFP (Fig. 1C, panel a), and subcutaneously injected into the flanks of athymic nude mice, apparent bony masses were formed in Ad-BMP9 transduced iCP15 and iCP18, while no masses were detected in the Ad-GFP infection groups (Fig. 1C, panel b). Micro-CT imaging analysis further confirmed ectopic bone formation (Fig. 1C, panel c). Hematoxylin and eosin (H&E) histologic analysis revealed the formation of mature and well-mineralized trabecular bone in both iCP15 and iCP18 cells stimulated with BMP9 (Fig. 1D, panel a; Fig. S2, panels a & b), which was further validated by Masson's trichrome staining (Fig. 1D, panel b). Collectively, these *in vitro* and *in vivo* findings have demonstrated that the SV40 LTA immortalized mouse cardiomyocyte progenitors are able to undergo effective osteoblast lineage-specific differentiation upon BMP9 stimulation, strongly suggesting that the iCP cells may possess MSC-like characteristics.

Mechanistically, we examined whether BMP9 could regulate the expression of critical osteogenic regulators in iCP15 and iCP18 cells, and found that the expression of both *Runx2* and *Osx* was significantly up-regulated at 36 h after Ad-BMP9 infection (Fig. 1E). Furthermore, BMP9 stimulation (at 72 h) significantly decreased the expression of the induced pluripotency stem cell (iPSC) factors *Oct4*, *Klf4* and *Sox2* in iPC15 and iPC18 cells (Fig. 1F, panels a & b), suggesting that BMP9-drived osteoblastic lineage-specific differentiation may reduce the SV40 LTA-induced pluripotency of the iCP cells. Accordingly, BMP9-driven osteogenic differentiation (at 120 h) markedly decreased the expression of mature cardiomyocyte markers *Anf*, *Tnnts*, and *Mhc* in both iCP15 and iCP18 cells (Fig. 1G, panels a and b). These results indicate that BMP9-driven osteogenic differentiation was involved in the up-regulation of osteogenic regulators Runx2 and Osx and the down-regulation of iPSC factors and cardiomyogenic differentiation in iCP15 and iCP18 cells.

Our findings have provided strong experimental evidence about a potential mechanism underlying SV40 LTA-mediated immortalization of mammalian primary cells. It has been well recognized that reduced p53 tumor suppressor activity increases the reprogramming efficiency of somatic cells.[3] It is thus conceivable that SV40 LTA may bind to and inhibit p53 on one hand, and transcriptionally regulate downstream target genes that exert similar or overlapping functions as that of the four iPSC factors, ultimately leading to at least a partial reprogramming phenotype of primary cells. Nonetheless, there are many missing links between SV40 LTA-induced immortalization and somatic cell reprogramming, and thus more in-depth investigations on their potential mechanistic connections are warranted.

In conclusion, we demonstrated that SV40 LTA-induced immortalization of the CPs derived from mouse E15.5 fetal heart was reversible by CRE recombinase. The iCP cells were able to undergo osteogenic differentiation both *in vitro* and *in vivo* upon BMP9 stimulation, a hallmark of multi-lineage features of mesenchymal stem cells (MSCs). Mechanistically, we found that BMP9-driven osteoblast lineage-specific differentiation was involved in the up-regulation of critical osteogenic regulators and the down-regulation of pluripotency factors and cardiomyogenic

mineralization medium. Alizarin Red S staining was conducted at day 9 and 11 after infection and observed under light microscope (×200). Representative results are shown. **(C)** BMP9-induced ectopic bone formation of iCP cells. Subconfluent iCP15 and iCP18 cells were infected with Ad-BMP9 or Ad-GFP for 24 h and observed under fluorescence microscope (×200) (**a**, only Ad-BMP9 shown), and harvested for subcutaneous injection into the flanks of athymic nude mice. At four weeks after injection, bony masses were retrieved from the Ad-BMP9 groups **(b)**, while no masses were detectable in the Ad-GFP groups. The retrieved bony masses were subjected to microCT imaging analysis. Representative images are shown **(c)**. **(D)** Histologic and special stains. The retrieved bony masses were fixed, decalcified and paraffin embedded. The tissue blocks were sectioned and subjected to H&E staining **(a)** and trichrome staining **(b)** and observed under light microscope (×400). H&E staining of more bony masses retrieved from different mice are shown in Figure S2. Representative results are shown. **(E)** BMP9-induced expression of osteogenic regulators in the iCP cells. Subconfluent iCP15 and iCP18 cells were infected with Ad-BMP9 or Ad-GFP. At 36 h post infection, total RNA was isolated and subjected to TqPCR analysis to determine the expression levels of *Runx2* and *Osx*. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the respective GFP groups **(F)** Downregulated expression of iPSC reprogramming factors during BMP9-induced osteogenic differentiation of the iCP cells. Subconfluent iCP15 **(a)** and iCP18 **(b)** cells were infected with Ad-BMP9 or Ad-GFP. At 72 h post infection, total RNA was isolated and subjected to TqPCR analysis to determine the expression levels of *Oct4*, *Klf4* and *Sox2*. $^{**}P < 0.01$, versus the respective GFP groups **(G)** Downregulated expression of cardiomyocyte mature markers during BMP9-induced osteogenic differentiation of the iCP cells. Subconfluent iCP15 **(a)** and iCP18 **(b)** cells were infected with Ad-BMP9 or Ad-GFP. At 120 h post infection, total RNA was isolated and subjected to TqPCR analysis to determine the expression levels of atrial natriuretic factor (*Anf*), cardiac troponin T (*Tnnt2*) and α-myosin heavy chain (*Mhc*). $^{*}P < 0.05$, $^{**}P < 0.01$, versus the respective GFP groups. All samples were normalized to *Gapdh* expression. All TqPCR reactions were performed in triplicate.

differentiation pathway. Collectively, our findings suggest the SV40 LTA-induced immortalization may resemble the reprogramming process of somatic cells although further in-depth investigations are highly warranted.

Conflict of interests

The authors declare no conflict of interests.

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Data availability statement

All datasets generated for this study are included in the manuscript and/or the Supplementary Material. Any further inquiries about data and resource availability can be directed to the corresponding authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.008.

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