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Induced Pluripotent Stem Cells to Model Human Fibrodysplasia Ossificans Progressiva

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SUMMARY

Fibrodysplasia ossificans progressiva (FOP) is a rare disease characterized by progressive ossification of soft tissues, for which there is no effective treatment. Mutations in the bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase 2 (ACVR1/ALK2) are the main cause of FOP. We generated human induced pluripotent stem cells (hiPSCs) from FOP patients with the ALK2 R206H mutation. The mutant *ALK2* gene changed differentiation efficiencies of hiPSCs into FOP bone-forming progenitors: endothelial cells (ECs) and pericytes. ECs from FOP hiPSCs showed reduced expression of vascular endothelial growth factor receptor 2 and could transform into mesenchymal cells through endothelial-mesenchymal transition. Increased mineralization of pericytes from FOP hiPSCs could be partly inhibited by the ALK2 kinase inhibitor LDN-212854. Thus, differentiated FOP hiPSCs recapitulate some aspects of the disease phenotype in vitro, and they could be instrumental in further elucidating underlying mechanisms of FOP and development of therapeutic drug candidates.

INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is an autosomal dominant genetic disorder in which acute inflammation may trigger the formation of a second skeleton of heterotopic bone. Classic FOP is caused by gain-of-function mutation (617G > A; R206H) in the activin receptor-like kinase 2 (ACVR1/ALK2) gene, encoding the bone morphogenetic protein (BMP) type I receptor (Shore et al., 2006). Enhanced BMP signaling in patients with the ALK2 R206H mutation has been attributed to loss of inhibitory activity of the ALK2-interacting protein FK506-binding protein-12 (FKBP12) (Chaikuad et al., 2012; van Dinther et al., 2010). Previous publications indicated that Tie2$^+$ endothelial cells (ECs) and mesenchymal cells (MCs) contributed as progenitor cells to the episodic heterotopic ossification (HO) in FOP (Medici et al., 2010; Wosczyna et al., 2012). Other cells like circulating osteogenic precursors, skeletal myoblasts, and vascular smooth muscle cells also were found in FOP lesions and may contribute to HO in FOP (Hegyi et al., 2003; Lounev et al., 2009; Suda et al., 2009).

Despite recent advances in understanding of the disease (Hatsell et al., 2015), to date there is no cure or even treatment for HO in FOP. A comprehensive understanding of the molecular mechanisms underlying abnormal behavior of bone-forming progenitor cells in FOP could be one approach toward effective treatment for HO in FOP, and to other more prevalent situations with HO that, for example, may occur after traumatic accidents or deep tissue burns. The traditional way of obtaining human biopsy tissues from FOP patients is limited as physical and surgical injury can induce HO. New protocols to produce well-characterized FOP bone-forming progenitor cells for research and therapeutic drug screening are needed. The ability to generate human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007) from adult tissues provides new opportunities for research on FOP. If derived from patients with genetic disease, hiPSCs allow production of large numbers of diseased target cells for basic research and drug development since they are immortal and pluripotent (Sterneckert et al., 2014).

In this study, we generated FOP hiPSCs from kidney cells isolated from urine by episomal vectors. The expression of pluripotent markers and ability to form derivatives of the three germ layers were comparable in FOP and control hiPSCs. However, the mutation in ALK2 reduced the efficiency of differentiation of hiPSCs into ECs and affected the phenotypes of ECs and pericytes. The hiPSC-derived ECs (hiPSC-ECs) from FOP patients exhibited reduced expression of vascular endothelial growth factor receptor 2 (VEGFR2) and could be transformed into MCs through endothelial-mesenchymal transition (EndMT). The hiPSC-derived pericytes (hiPSC-pericytes) from the FOP group showed increased ability to mineralize compared with the

control. Our experiments demonstrated that disease-relevant cells differentiated from FOP hiPSCs possessed phenotypes reminiscent of the FOP disease.

RESULTS

Generation of FOP hiPSCs from Urine Cells

We used a rapid and non-invasive procedure to isolate kidney cells in urine from FOP patients (Xue et al., 2013). The cells were isolated from 50–100 ml middle stream of the micturition from two male FOP patients (Dutch and Chinese, F2 and F3) diagnosed with the classic R206H mutation and two healthy male donors (Dutch and Chinese, C2 and C3) (Figure S1B).

A schematic representation of hiPSC generation is shown in Figure S1A. In summary, cultured cells from urine were electroporated with episomal vectors containing *OCT4*, *SOX2*, *KLF4*, and the *pCEP4-miR-302-367* cluster (containing *miR-302b*, *c*, *a*, *d*, and *miR-367*) (Xue et al., 2013). Transfected urine cells were maintained in serum-free mTesR1 medium supplemented with a cocktail of small molecule inhibitors to promote reprogramming: CHIR99021, PD0325901, A83-01, and thiazovivin (Wang et al., 2013). Small colonies of cells appeared that progressively adopted a human embryonic stem cell (hESC)-like morphology. Selected hiPSCs were picked manually and expanded at day 20; hiPSCs maintained their hESC-like morphology with prominent nuclei and little cytoplasm and stained positive for alkaline phosphatase (ALP) (Figure S1B).

The hiPSCs from one healthy donor (C3) and from two FOP patients (F2 and F3) were characterized; the other control hiPSC line (UE017C1) was obtained from the Guangzhou Stem Cell Bank produced by the same method and was characterized previously (Xue et al., 2013). The presence of classical mutation in the *ALK2* gene was confirmed in FOP hiPSCs (Figure S1C). FOP and control hiPSC karyotypes were checked before passage 10 and these were normal (Figure S1D). The loss of exogenous reprogramming factors and episomal backbones was demonstrated by genomic PCR that specifically amplifies exogenous factors (Figure S1E). The quantitative real-time PCR analysis revealed that, compared to urine cells, FOP hiPSCs had upregulated expression of endogenous hESC transcriptional genes (endogenous *OCT4*, endogenous *SOX2*, *NANOG*, and *REX1*), and they had expression levels comparable with established H1 hESCs (Figure S1F). Immunofluorescence microscopy showed expression of pluripotency-associated antigens OCT4, SSEA-4, TRA-1-60, and TRA-1-81 (Figure S1G). In addition, hiPSCs formed teratomas in mice in vivo, confirming the pluripotency of FOP iPSCs (Figure S1H). Therefore, hiPSCs from FOP patients corresponded phenotypically and functionally to hESCs.

Impaired EC Differentiation of FOP hiPSCs

A slight elevation of pSMAD1/5 signaling in FOP hiPSCs compared to control hiPSCs was observed when these cells were cultured for 24 hr in medium with low serum concentrations, but not in the undifferentiated FOP iPSCs (Figures 1A and S1I). We differentiated hiPSCs into ECs and pericytes to examine how minor changes in ALK2 R206H/SMAD signaling influenced the fate of two possible progenitor cells of FOP, i.e., ECs and pericytes. On days 10–12 of differentiation, flow cytometry (fluorescence-activated cell sorting [FACS]) analysis showed that two cell populations formed as follows: CD31$^+$ ECs and platelet-derived growth factor receptor (PDGFR) β$^+$ pericytes (Figure 1B). The generation of CD31$^+$/VE-cadherin$^+$ cells was significantly impaired in FOP hiPSCs compared with controls, while general mesoderm induction was slightly enhanced in FOP hiPSCs (Figures 1B and 1C).

To verify the FACS data, we examined the expression of early mesoderm and EC-specific genes (Figure 1D). Differentiation resulted in efficient downregulation of pluripotent markers (*OCT4* and *NANOG*) in the control and FOP hiPSCs. Primitive streak/mesoderm lineage markers (*T* and *PDGFRα*) were upregulated in FOP hiPSCs on day 4 of differentiation, which may due to the positive effect of the BMP-signaling pathway on mesoderm formation. The induction of the early endothelial transcription factor (*ETV2*) on day 4 was similar between the two groups. However, consistent with the FACS data, we observed that endothelial-specific genes (*CD31* and *CD105*) were downregulated in FOP hiPSCs on day 7 of differentiation. Early pericyte markers *PDGFRβ* and *NG2* proteoglycan were expressed more abundantly in differentiating FOP hiPSCs. Overall, we observed that the EC differentiation efficiency was impaired while general mesoderm differentiation was enhanced in FOP hiPSCs; this difference may due to the elevated level of ALK2 R206H/SMAD signaling in FOP compared to control hiPSCs.

Characterization of FOP hiPSC-ECs

Differentiated cell populations were divided into CD31$^+$ ECs and CD31$^-$ cells by CD31 antibody-coupled magnetic bead sorting (Figure 2A). The expression levels of BMP type I receptors ALK1 and ALK2 were not different in control versus FOP CD31$^+$ cells (Figure S2A). Sorted FOP hiPSC-ECs were more sensitive to low concentrations of BMP6 (5 ng/ml) and the activated BMP signaling could be inhibited by BMP type I receptor kinase inhibitor LDN-193189 (Yu et al., 2008; Figure 2B). FOP hiPSC-ECs exhibited poor viability and increased expression of senescence-associated β-galactosidase expression compared to controls (Figures S2B and S2C). As the VEGF-signaling pathway is known to regulate survival and proliferation of ECs through VEGFR2 (Kelly and Hirschi, 2009), we

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