Expression of Normal or Mutated X-Linked *BCOR* Transcripts in OFCD iPSCs

Journal of Dental Research
2020, Vol. 99(2) 196–203
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for Dental Research 2019
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DOI: 10.1177/0022034519890323
journals.sagepub.com/home/jdr

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Abstract

Reprogramming diseased cells with mutated genes into induced pluripotent stem cells (iPSCs) can allow studies of disease mechanism and correct the mutation. Oculofaciocardiodental (OFCD) syndrome is a developmental disorder caused by heterozygous mutations in the X-linked BCL-6 corepressor (*BCOR*) gene. In this present study, we aimed to reprogram stem cells from a tooth apical papilla (SCAP) of a patient with OFCD, termed SCAP-O, into iPSCs. The SCAP-O carry a copy of the *BCOR* gene having 1 nucleotide deletion in 1 of the alleles, therefore harboring a mixture of cells expressing either normal (SCAP-O^{BCOR-WT}) or mutated (SCAP-O^{BCOR-mut}) *BCOR* transcripts. We subcloned SCAP-O and separated SCAP-O^{BCOR-WT} and SCAP-O^{BCOR-mut} as verified by sequencing. The selected subclone SCAP-O^{BCOR-mut} expressed only the mutated *BCOR* transcripts and remained in such condition after multiple passages. We reprogrammed SCAP-O and subclone SCAP-O^{BCOR-mut} into transgene-free iPSCs using an excisable lentiviral vector system (hSTEMCCA-loxP) carrying 4 reprogramming factors in a single cassette, followed by removal of transgenes via Cre-mediated excision. We found that after reprogramming SCAP-O or subclone SCAP-O^{BCOR-mut} into iPSCs, some of the iPSC clones expressed either solely the normal *BCOR*-WT or *BCOR*-mut transcripts, while other clones expressed both *BCOR*-WT and *BCOR*-mut transcripts. This is our first step toward establishing OFCD study models by generating isogenic control *BCOR*-WT iPSCs versus *BCOR*-mut iPSCs.

Keywords: induced pluripotent stem cells, reprogramming, oculofaciocardiodental syndrome, stem cells of apical papilla, BCL-6 corepressor gene, X-linked dominant syndrome

Introduction

Induced pluripotent stem cells (iPSCs) generated from diseased cells may aid researchers in studying the underlying mechanisms of the disease or for drug development (Nishikawa et al. 2008; Park et al. 2008; Jang et al. 2012; Kumar et al. 2018). Oculofaciocardiodental (OFCD) syndrome, caused by the mutation in the BCL-6 corepressor (*BCOR*) gene (Hedera and Gorski 2003; Horn et al. 2005; Oberoi et al. 2005; Di Stefano et al. 2015; O’Byrne et al. 2017), typically presents the following clinical characteristics: multiple minor craniofacial deformations or defects, microphthalmia, congenital cataracts, and congenital heart disease. OFCD is an X-linked dominant trait in heterozygous female patients, and males cannot survive from the embryonic lethality (Hedera and Gorski 2003). Of those characteristics that are more related to dental conditions, patients with OFCD may show various dental anomalies, including prolonged retention of primary teeth, delayed eruption of permanent teeth, oligodontia, radiculomegaly of canine teeth, and dilaceration of roots (Oberoi et al. 2005). The *BCOR* sequence of a patient with OFCD was found to have a single-nucleotide deletion, c.2613delC, creating a frameshift mutation and premature stop codon (Oberoi et al. 2005). Stem cells from apical papilla (SCAP) were isolated from this patient, termed SCAP-O. These cells appeared to show stronger production of mineral tissue in vivo when they were mixed with

hydroxyapatite/tricalcium phosphate and transplanted into the dorsal subcutaneous space of immunocompromised mice (Fan et al. 2009). These SCAP-O were previously clonally sequenced and found to have 87% of the cell population expressing the mutated *BCOR* copy (*BCOR*-mut; Fan et al. 2009). However, cells from patients with OFCD have not been reprogrammed into iPSCs, which may be used as a model to further study this disease. Generated OFCD iPSCs have the following advantages for studies: 1) they are immortalized cells and have the potential to be propagated unlimitedly; 2) they can be differentiated into any desired cell types for various mechanistic studies; and 3) since OFCD is an X-linked

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A supplemental appendix to this article is available online.

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dominant trait, we have the potential to isolate the isogenic control normal counterpart for comparison studies. In this present study, we aimed to reprogram SCAP-O into transgene-free (TF) iPSCs as the first step and to determine whether iPSC clones express the *BCOR*-mut and/or *BCOR*-WT transcripts to provide a basis for whether we can generate isogenic control OFCD iPSCs (normal counterpart) and establish an OFCD iPSC model for future studies.

Materials and Methods

Cell Culture

The SCAP-O cultures were established previously (Fan et al. 2009; obtained from Dr. Songtao Shi, University of Southern California; original protocol following University of California San Francisco Institutional Review Board [IRB] with informed patient consent). The study of SCAP-O was approved by the IRB of the University of Tennessee Health Science Center (UTHSC; 19-06855-NHSR) as not human subjects research. SCAP-WT (wild type) were obtained from freshly extracted immature human teeth from normal individuals as described before (Zou et al. 2012). The wild-type tooth sample collection was based on approved exempt protocols by the IRB of Boston University (H-28882) and UTHSC (12-01937-XM). Cells were maintained in α -Minimum Essential Medium (GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc.), 2mM L-glutamine, 100 μ M L-ascorbic acid 2-phosphate, 100-U/mL penicillin-G, 100- μ g/mL streptomycin, and 0.25- μ g/mL Fungizone (Gemini Bio-Products, Inc.) and maintained in 5% CO₂ at 37 °C. Feeder cells were mouse embryonic fibroblasts obtained from ATCC or GlobalStem, Inc. or isolated directly from E13.5 embryos of CF1 pregnant mice according to the National Institutes of Health's standard protocol (<http://stemcells.nih.gov/research/nihresearch/scunit/culture.htm>) and cultured in DMEM supplemented with 10% fetal bovine serum (Zou et al. 2012; El Ayachi et al. 2018). All animal procedures followed approved protocols (Institutional Animal Care and Use Committee at Boston University, protocol AN15026; at UTHSC, protocol 2082). For culturing iPSCs, human embryonic stem cell (hESC) medium (80% DMEM/F12, 20% knockout serum replacement, 1 $\times$ nonessential amino acid, 1mM L-glutamine, 0.1 μ M β -mercaptoethanol) containing 4-ng/mL basic fibroblast growth factor was used.

Subcloning of SCAP^{BCOR-mut}

The SCAP-O carry a copy of the mutated *BCOR* gene in 1 of the alleles, therefore harboring a mixture of cells expressing either normal (SCAP-O^{BCOR-WT}) or mutated (SCAP-O^{BCOR-mut}) *BCOR* transcripts. These heterogeneous SCAP-O at passage 7 were diluted and seeded into a 96-well plate such that each well received 1 cell. An emerged single colony in each well was then passaged and extended into 12-well plates. These subclones were expanded until enough cells were reached for sequencing and the following generation of iPSCs. Each

expanded SCAP-O subclone was harvested for RNA isolation and sequencing analysis (GENEWIZ, Inc.).

Transduction and Reprogramming of SCAP-O

Lentiviral vector set pSin-EF2-gene-Pur (each carrying 1 of the 4 human factors *LIN28*, *NANOG*, *OCT4*, and *SOX2*) was first used to reprogram SCAP-O as described previously (Yu et al. 2007; Yan et al. 2010). To generate TF iPSCs, lentiviral vectors hSTEMCCA-LoxP (a polycistronic single vector carrying all 4 human reprogramming factors: *c-MYC*, *KLF4*, *OCT4*, and *SOX2*) were also used to reprogram these cells, and the excision of hSTEMCCA was subsequently performed as described in our previous reports (Zou et al. 2012; El Ayachi et al. 2018). After transgene excision, genomic DNA from each selected SCAP-O iPSC clone was extracted with Quick-gDNA MicroPrep kit (D3020; Zymo Research) and polymerase chain reaction (PCR) performed to verify the excision of the hSTEMCCA. The primers used and the PCR conditions are as follows: *c-MYC* (GenBank accession NM_002467.6), forward primer: 5'-GGA ACT CTT GTG CGT AAG TCG ATA G-3', *WPPE* (reverse primer): 5'-GGA GGC GGC CCA AAG GGA GGA GAT CCG-3'; *GAPDH* (GenBank accession NM_002046), forward primer: 5'-CAA GGC TGA GAA CGG GAA GC-3'; reverse primer: 5'-AGG GGG CAG AGA TGA TGA CC-3'; 95 °C, 3 min, followed by 33 cycles of 94°C, 30 s, 60°C, 30 s, and 72°C, 5 min. The PCR products were examined by electrophoresis on an agarose gel. Verified TF clones were named TF SCAP-O iPSCs. To verify that there was no integration of pHAGE2-Cre-IRES-PuroR plasmid DNA into the genome of TF SCAP-O iPSCs, these cells were grown on DR4 mouse embryonic fibroblasts in the presence of puromycin (1.2 μ g/mL) for up to a week. An inactive fragment of the viral long terminal repeat remains in the host genome after Cre excision (Zou et al. 2012). Data presented in Figures 1 to 5 and Appendix Figure 2 were derived from SCAP-O iPSCs generated via hSTEMCCA/Cre system.

Immunocytofluorescence

See Appendix.

Teratomas Formation

See Appendix.

Sequencing

For cDNA sequencing, total RNA was extracted from cultured cells with the RNeasy Mini Kit (74106; Qiagen) and DNase I (Invitrogen) to remove genomic DNA contaminants. RNA (1 μ g) was used for first-strand cDNA synthesis with the SuperScript III RT-PCR Kit (Invitrogen). For gDNA sequencing, genomic DNA was extracted from cultured cells (Zou et al. 2012; El Ayachi et al. 2018). PCR was performed with the specific primers that spanned the mutation site of *BCOR*. The primers

Figure 1. Generation of transgene-free SCAP-O iPSCs. **(A)** Emergence of SCAP-O iPSCs during reprogramming. **(Aa)** SCAP-O at passage 4 (p4) before transduction (40 $\times$). **(Ab)** SCAP-O at p5 were transduced with hSTEMCCA-loxP lentiviral vector and seeded onto MEFs. A small ESC-like colony emerged on day 23 posttransduction (40 $\times$). **(Ac)** A ESC-like colony on day 23 posttransduction on MEFs. The ESC-like colony is considered at p0. **(B)** Excision of hSTEMCCA. **(Ba)** Established SCAP-O iPSC colonies at p2 before transgene excision. **(Bb)** After pHAGE2-Cre-IRES-PuroR plasmid transfection and 48 h of puromycin treatment, most SCAP-O iPSC colonies died out, leaving behind an empty space (arrow). **(Bc)** New colonies (arrow) emerged on day 1 after Cre excision and puromycin selection. **(Bd)** Expanded SCAP-O iPSC colonies passaged twice after Cre excision. **(C)** Excision of hSTEMCCA was verified by reverse transcription polymerase chain reaction. Clones after Cre excision showed no detectable hSTEMCCA. GAPDH served as a control. **(D)** Verification of Cre-free status of SCAP-O iPSC. **(Da, b)** Before treatment of SCAP-O iPSCs seeded on DR4 MEF with puromycin. **(Dc, d)** After treatment with puromycin for 48 h, showing cell death. Scale bars: Aa, Ab, B, Da, Dc, 500 μ m; Ac, 200 μ m; Db, Dd, 250 μ m. ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; MEFs, mouse embryonic fibroblasts; SCAP-O, stem cells from apical papilla of a patient with oculofaciocardiodental syndrome.

for *BCOR* were as follows: GenBank accession NM_001123385.2, forward: 5'-CCC CAA CTG GAA TCA AGG GAA GAC-3', reverse: 5'-TAA CCC GCT GAG TTG GCG ATT C-3'; target size, 552 bp. The PCR product was analyzed by electrophoresis on 1.5% agarose gel and sent to GENEWIZ for sequencing following the procedure (<https://www.genewiz.com/Public/Services/Sanger-Sequencing/PCR-Purification>). The sequencing was performed with *BCOR* primers as used for PCR.

Embryoid Body–Mediated Neurogenic Differentiation In Vitro

This method was based on our previous report (Zou et al. 2012). Briefly, TF iPSC colonies grown on Matrigel/TESR2

medium (Stemcell Technology) were detached and placed into ultra-low 6-well plates for embryoid body (EB) formation. After 4 d, EBs were collected and plated into Matrigel-coated wells of 6-well plates. After EB attachment, the medium was changed to neurogenic differentiation medium 1 (DMEM supplemented with B27 and 20-ng/mL basic fibroblast growth factor) and cultures incubated for 8 d before being fixed and stained for neural markers β III-tubulin, nestin, and neurofilament medium chain.

Results

SCAP-O Reprogrammed into iPSCs

We first used lentiviral vectors from the pSin-EF2-gene-Pur (*LIN28/NANOG/OCT4/SOX2*) system to generate iPSCs from

Figure 2. In vitro neural differentiation potential and teratoma formation of TF SCAP-O iPSCs. **(A)** Embryoid body-mediated neural differentiation. (Aa, b) TF SCAP-O iPSCs were cultured on Matrigel. (Ac–f) The attached embryoid bodies formed neural rosettes under neural induction for 5 d. Some cells extended elongated cell cytoplasmic processes resembling neurons. **(B)** Immunocytofluorescence analysis of differentiated neural-like cells shows positive staining of neural marker β III-tubulin. Cells forming a neural rosette or spherical morphology were also positive for nestin and CNPase. Control: isotype antibody control. Expressed genes stained in red; DAPI, nuclear stain. DAPI, 4',6-diamidino-2-phenylindole. **(C)** Histologic analysis of teratomas formed by SCAP-O iPSCs (passage 11) before and after Cre excision. (Ca, b, i, j) Lower-magnification views illustrate tissues representing all 3 germ layers. (Cc, d, k, l) Mainly neural tissues, neural rosettes (red arrows), and neural tube-like (blue arrows) (ectoderm). (Ce, f, m, n) Mainly cartilage (yellow arrows) or bone (green arrows) (mesoderm), (Cg, h, o, p) Mainly glandular epithelium (white arrows) and endodermal tissues. Scale bars: Aa, B, top panel β III-tub, Ca, Cb, Ci, Cj, 500 μ m; Ab, Ad, Ae, Af, B, second from top β III-tub, CNPase, control, 200 μ m; Ac, B, nestin, Cc–Ch, Ck–Cp, 100 μ m. iPSCs, induced pluripotent stem cells; SCAP-O, stem cells from apical papilla of a patient with oculofaciocardiodental syndrome; TF transgene-free.

Figure 3. Sequence analysis of *BCOR* mRNA expressed in TF SCAP-O iPSCs. Five clones of the iPSCs were harvested for gDNA isolation or RNA isolation (converted into cDNA) and sequenced. Results of 4 clones are represented. **(A)** gDNA sequencing of the *BCOR* gene shows known mutation site TTT(C)AAA (C nucleotide is boxed). All 5 clones showed both alleles of the gDNA *BCOR* gene—one allele with deleted C nucleotide and the other allele with the normal sequence. The sequence readout in panel Ab shows the deletion of the C nucleotide (black arrowhead) from the automated reading. Red arrows show the 2 groups of readout nucleotide peaks after the C deletion site. **(B)** The mRNA of *BCOR* (cDNA analysis) of the corresponding clones in panel A were also sequenced, and all but 1 clone expressed only the normal *BCOR* mRNA. The 1 clone exhibited a mixture in the *BCOR* mRNA pool—that either the C is deleted or not deleted, resembling the gDNA sequence chromatogram result. Black arrows indicate that the C nucleotide is present; red arrows, the shifted peaks of nucleotide sequences due to the missing C nucleotide. iPSCs, induced pluripotent stem cells; SCAP-O, stem cells from apical papilla of a patient with oculofaciocardiodental syndrome; TF, transgene-free.

SCAP-O. We found that SCAP-O appeared to be more difficult to reprogram than our earlier experiments reprogramming SCAP from healthy individuals (Yan et al. 2010). SCAP-O were transduced with virus 3 times before seeded onto the feeder cells for reprogramming. We obtained 22 ESC-like colonies from SCAP-O after transduction. These reprogrammed SCAP-O iPSCs exhibited a morphology indistinguishable from hESCs in cultures and formed EBs with cystic cavities when cell colonies were grown in a floating condition (Appendix Fig. 1A; also see Appendix Materials and Methods). SCAP-O iPSCs expressed hESC-associated markers OCT4, SOX2, SSEA-4, TRA-1-60, and TRA-1-81 (Appendix Fig. 1B). After reattaching the EBs in cultures, the cells spontaneously differentiated into cells representing all 3 germ layers expressing markers AFP (endoderm), β III tubulin and GFAP (ectoderm), α -SMA (mesoderm), and vimentin (mesoderm and parietal endoderm), having a similar capacity to SCAP-WT iPSCs generated from

cells of a healthy individual (Appendix Fig. 1C, D).

We then generated TF SCAP-O iPSCs using lentiviral vectors hSTEMCCA-LoxP, followed by treatment with Cre-IRES-PuroR plasmids to remove the transgenes based on our previous reports (Zou et al. 2012; El Ayachi et al. 2018). This transduction system appeared more efficient to transduce SCAP-O than the aforementioned method. With SCAP-O iPSCs from different clones, removal of hSTEMCCA vector was found in 100% of these newly emerged subclones (Fig. 1A–C). TF SCAP-O iPSCs were verified for the absence of the integration of pHAGE2-Cre-IRES-PuroR plasmid DNA into the genome (Fig. 1D).

After generation of SCAP-O iPSCs with the hSTEMCCA system, we examined their in vitro neurogenesis capacity and in vivo teratoma formation. As presented in Figure 2A, EB-mediated neural differentiation of TF SCAP-O iPSCs formed neural rosettes or neural tube-like structures in cultures, and some cells displayed neuron-like phenotype morphologically in only 5 d of neural induction. They also expressed basic neural markers— β III-tubulin, nestin, and CNPase (Fig. 2B)—that we examined by immunocytofluorescence analysis. They were also able to form teratomas before or after Cre excision of the hSTEMCCA transgenes. The teratomas contained primitive ectodermal (neural tissues), mesodermal (cartilage and bone), and endodermal tissues (glandular epithelium and tissues; Fig. 2C).

The rest of the data presentation (Figs. 3–5, Appendix Fig. 2) is based on studies of TF SCAP-O iPSCs.

TF SCAP-O iPSCs Expressed Normal Copies of *BCOR* mRNA

We then examined the *BCOR* sequence from these generated TF SCAP-O iPSCs at the gDNA and mRNA levels. Due to this single-nucleotide deletion in one of the *BCOR* genes, we can use the sequencing chromatogram to observe 2 mixed populations of sequence, as shown in Figure 3 (5 clones were subjected to analysis; 4 are shown). The gDNA sequencing of the TF SCAP-O iPSC clones presented in Figure 3A indicates a shift of peaks at the C nucleotide in the TTT-C-AAA region, confirming that the gDNA sample contained *BCOR* genes from both alleles (normal and mutated). Depending on which *BCOR* PCR products gave a stronger peak, the automated readout would report either normal or mutated sequence.

The same clones used to sequence gDNA were used to generate cDNA (from mRNA), and the PCR products were sequenced as shown in Figure 3B (showing 4 of 5 studied). None of the 5 clones expressed the deleted *BCOR* mRNA based on the automated sequence readouts. The nucleotide peaks in Figure 3Bb, however, showed that this 1 clone has a mixed population of normal and mutated *BCOR* transcripts. By the intensity of the peaks, the mutated *BCOR* appears to be at low levels, almost close to the noise background. Such intensity of the peaks may not be used as a quantitative mean to determine which copy of the *BCOR* mRNA population is the dominant one; however, it may be a qualitative indicator regarding which transcript has more copies in the mRNA pool. Other clones showed only pure *BCOR*-WT transcripts.

Subcloning of SCAP-O

The SCAP-O original pool contained a mixture of SCAP-O expressing *BCOR*-WT or *BCOR*-mut mRNA (but not both) in the same cell due to random X chromosome inactivation (XCI). To determine the outcome of generated iPSCs from a pure population of a subclone of SCAP-O that expresses solely the mutated *BCOR* mRNA, we performed 2 independent experiments of subcloning SCAP-O. We first sequenced PCR products of *BCOR* from the cDNA of 4 subcultures of SCAP-O at different passages and batches. As expected, all 4 showed a mixed population of normal and mutated *BCOR* based on the nucleotide peak patterns, and the sequence automated readouts all reported a C deletion (Fig. 4A–D).

In the first round of subcloning, we selected and sequenced cDNA of 24 subclones. We found that 10 expressed the mutated *BCOR* transcripts, termed SCAP-O^{*BCOR*-mut} (with deleted C nucleotide), and 10 expressed the normal transcripts, termed SCAP-O^{*BCOR*-WT}. Representative subclone sequence results are shown in Figure 4E and F. One subclone expressed normal *BCOR* transcripts, and the other expressed the mutated. The nucleotide peak patterns after the TTTCAAA site displayed no signs of mixed population of *BCOR* transcripts, indicating that they are a pure population of subclone. Of the 21 clones successfully sequenced, 1 expressed mixed *BCOR* transcripts (mutated and normal), and the remaining clones were either not sequenced (grew too low) or sequenced unsuccessfully. About 6 SCAP-O^{*BCOR*-mut} subclones grew very slowly, and cells were larger than normal SCAP, possibly due to prolonged cell culture passaging or other unknown reasons.

In the second round of subcloning, 10 of 18 selected clones were sent for sequencing, and 7 expressed mutated *BCOR* and 3 normal. The first round of subcloning gave 50% of clones

Figure 4. cDNA sequence analysis of *BCOR* mRNA expressed in SCAP-O and their subclones. (A–D) Original pool of SCAP-O of different passages were subjected for sequencing analysis of the *BCOR* mutation segment. All 4 subcultures of SCAP-O showed a deleted C nucleotide from the sequence reading while revealing a mixed population of *BCOR* mRNAs, with both copies of normal *BCOR* sequence and the deleted C nucleotide sequence. Black arrowhead: deleted C nucleotide site. Blue arrows: the peak of the C nucleotide present in the other populations of the *BCOR* mRNA. Red arrows: the shifted peaks of the nucleotide sequences representing the normal *BCOR* mRNA transcripts. (E, F) Sequence analysis of subclones from SCAP-O pooled population. The polymerase chain reaction product of the cDNA from 21 subclones out of 24 had successful sequencing results. Representing sequence data shown here: (E) a subclone expressing pure normal *BCOR* transcripts and (F) a subclone expressing pure mutated *BCOR* transcripts. SCAP-O, stem cells from apical papilla of a patient with oculofaciocardiodental syndrome.

expressing the mutated gene, and the second round of subcloning gave 70% of clones expressing the mutated gene. Together, an average of 57% of SCAP-O expressed the mutated *BCOR*.

Reactivation of the Normal *BCOR* Transcript after Reprogramming

We selected 2 SCAP-O^{*BCOR*-mut} subclones for reprogramming using hSTEMCCA-LoxP vectors. We also verified that these SCAP-O^{*BCOR*-mut} subclones continued to express the *BCOR*-mut transcript after cell passaging. Two iPSC clones emerged from 1 of the 2 SCAP-O^{*BCOR*-mut} clones. They were expanded and the RNA isolated for *BCOR* sequencing. We found that these iPSCs expressed normal and mutated *BCOR* transcripts, as shown in Figure 5A. Subsequently, their transgenes were removed by Cre-mediated excision and validated via PCR examination of the gDNA. These iPSC clones were named TF SCAP-O^{*BCOR*-mut} iPSCs. We then selected 4 clones for sequencing analysis, and the results showed that only 1 clone expressed the pure mutated *BCOR* transcripts. The rest were mixed with normal and muted *BCOR* transcripts upon examination of the sequencing peaks of the nucleotide despite the readouts showing 3 of 4 as the mutated transcript (Fig. 5B).

We further selected and sequenced 6 clones of the TF SCAP-O^{*BCOR*-mut} iPSCs to verify the expression condition. We found that all 6 iPSC clones sequenced displayed mixed

Figure 5. cDNA sequence analysis of *BCOR* mRNA expressed in SCAP-O^{BCOR-mut} iPSCs reprogrammed from subclones SCAP-O^{BCOR-mut}. **(A)** The cDNA of 2 clones of SCAP-O^{BCOR-mut} iPSCs before excision of hSTEMCCA transgenes was sequenced for the *BCOR* gene, showing mutated and normal *BCOR* transcripts (mixed). The gene sequence readout indicated that both subclones have a C nucleotide deleted (black arrowhead). **(B)** After excision of hSTEMCCA transgenes, the cDNA of 4 clones of TF SCAP-O^{BCOR-mut} iPSCs was sequenced for *BCOR* mRNA, showing pure mutated *BCOR* transcripts (Ba) or mixed (Bb–d) having both mutated and normal *BCOR* transcripts. Sequence readouts indicate only panel Bc has the normal sequence. Note: The sequence in panel Bd shows that the normal *BCOR* transcript peaks are close to background level. **(C)** Six more clones of the TF SCAP-O^{BCOR-mut} iPSCs were further sequenced to verify, and 2 representative results are presented herein. iPSCs, induced pluripotent stem cells; SCAP-O, stem cells from apical papilla of a patient with oculofaciocardiodental syndrome; TF, transgene-free.

expression of normal and mutated *BCOR* transcripts (2 representative results are in Fig. 5C). All these sequencing results showed that the normal *BCOR* transcripts appeared dominant; therefore, the sequence readouts all showed normal *BCOR*.

Discussion

Our results showed that heterogeneous SCAP-O can be reprogrammed into iPSCs that may express only the normal copy of *BCOR* or normal and mutated. Although further analysis on the XCI and XIST RNA status is the next step, the findings suggest that SCAP-O iPSCs potentially exhibit either the pre-XCI state (XaXa both alleles activated) or post-XCI state (XaXi, 1 allele activated, 1 inactivated). That TF iPSCs derived from subclone SCAP-O^{BCOR-mut} may express both *BCOR* copies or 1 of the 2 further suggests this phenomenon.

TF iPSCs reprogrammed from the mosaic population of SCAP-O expressed mainly *BCOR*-WT transcripts with 4 of 5 clones expressing pure *BCOR*-WT and with 1 clone expressing a mixture of *BCOR*-WT and *BCOR*-mut, with the former

predominant (Fig. 3Bb). TF iPSCs reprogrammed from SCAP-O^{BCOR-mut} gave rise to 1 (of 10 clones studied) that expressed solely the *BCOR*-mut transcripts, whereas the rest all expressed both, with either the *BCOR*-mut transcripts dominating (2 of 10) or *BCOR*-WT dominating (7 of 10; Fig. 5B, C). While total number of clones examined is not large, it appears that *BCOR*-WT transcripts tended to be dominantly expressed after reprogramming.

Due to random XCI at the early stage of embryonic development (Barakat and Gribnau 2012), OFCD is the result of the mutated *BCOR* copy that becomes active during and after differentiation in some population of cells. Per a previous report, 87% of the SCAP-O expressed the mutated *BCOR* copy and the rest of the cells expressed the normal transcripts (cell subcloning was not performed in this reported study; Fan et al. 2009). This mosaic population of SCAP-O was enough to show OFCD phenotypic characteristics in experimental settings, such as higher proliferative rate and levels of ALP activity, as well as stronger osteogenic potential in cultures as compared with SCAP-WT from normal individuals (Fan et al. 2009). We sequenced these mosaic SCAP-O of different passages and found that all showed expression of *BCOR*-WT and *BCOR*-mut transcripts, while the sequencing readouts indicated all samples representing *BCOR*-mut (Fig. 4A–D). As mentioned earlier, this sequencing chromatogram showed the 2 groups of nucleotide peaks after the C nucleotide of

TTTCAAA segment with the TTAA population as the predominant group. Our subcloning of the mosaic population of SCAP-O, followed by sequencing of individual subclones, yielded an average of 57% SCAP-O expressing *BCOR*-mut transcripts and the remaining expressing *BCOR*-WT. The percentage of SCAP-O expressing *BCOR*-mut is not as high as the reported 87% (Fan et al. 2009). This could be the result of subculturing over time and the change of these subpopulations, or a larger sample size was needed.

Well-reprogrammed iPSCs should exhibit similar XCI states to that of hESCs. The XCI status in hESCs can be either in a pre-XCI state displaying XCI characteristics upon differentiation or in the undifferentiated state having an inactive X chromosome (post-XCI state; Geens and Chuva De Sousa Lopes 2017). iPSCs from cells of patients with Rett syndrome (RTT), an X-linked neurodevelopmental disorder, have been studied (Cheung et al. 2012). Similar to OFCD, the disease affects girls with heterozygous mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2; Cheung et al. 2012). The XCI status of RTT-hiPSCs (i.e., human iPSCs)

has been reported to be either retaining the inactive X-chromosome of the founder somatic cells—allele-specific expression—or showing reactivation of the inactive X-chromosome in RTT-hiPSCs derived from the founder somatic cells (Cheung et al. 2012). The subsequent random XCI occurs when RTT-hiPSCs undergo differentiation, generating a mosaic cell population expressing either *MECP2*-WT or *MECP2*-mut transcripts. Our findings suggest that the XCI status of reprogrammed SCAP-O could be similar to what was found in the RTT-hiPSC model. We also examined 2 lineage differentiation pathways—osteogenesis and neurogenesis—to determine which *BCOR* transcript was expressed after iPSC differentiation into specific lineages of differentiated cells. Three of the sequenced TF SCAP-O^{BCOR-mut} iPSC clones were selected and subjected to the experimentation, and results are shown in Appendix Figure 2 (also see Appendix Materials and Methods). The *BCOR* transcript sequence analysis showed that *BCOR*-WT and *BCOR*-mut remained expressed in a combination similar to that before differentiation—with *BCOR*-WT transcript as the dominant copy. Further sequencing analysis at the cell clonal level after differentiation is needed.

The present study is the first step to establish an OFCD iPSC model that may generate an isogenic control cell population (*BCOR*-WT) and its diseased counterpart *BCOR*-mut for further mechanistic studies. Future functional studies based on this model, such as in vitro neural lineage functional studies and in vivo osteogenic tissue formation analysis, may also provide information regarding the effect of *BCOR* mutation on cell lineage differentiation.

Author Contributions

I. El Ayachi, X.-Y. Zou, contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; X. Yan, Y. Lou, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; G.T.-J. Huang, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

The authors thank Dr. Songtao Shi and his associates (University of Pennsylvania) for his input and assistance during the early developmental stage of the project; Dr. Darrell N. Kotton and his associates (Pulmonary Center and Department of Medicine, Boston University) for their assistance with TF reprogramming methods; Dr. Jun Zhang (UTHSC) for his assistance in iPSC cultures; and Kimberley Chan (Boston University) for assistance with immunostaining. This work was supported in part by a grant from the National Institutes of Health (RO1 DE019156, G.T.J.H.), a research fund from the Boston University School of Dental Medicine (G.T.J.H.), and a research fund from UTHSC (G.T.J.H.). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Compliance with Ethical Standards

Research involving human participants and/or animals: To-be-discarded extracted teeth were collected from clinics at Boston

University and UTHSC based on exempt protocols approved by the medical IRBs (Boston University, H-28882; UTHSC, 12-01937-XM, 19-06855-NHSR). No consent form was needed for the present study. All animal procedures followed an approved protocol (Institutional Animal Care and Use Committee at Boston University, protocol AN15026; UTHSC, protocol 2082).

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Data Sharing

Sharing cell lines: Available cell lines will be shared with other investigators upon request and after completing an institutional materials transfer agreement.

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