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

I. El Ayachi, X.-Y. Zou, X. Yan, Y. Lou, and G.T.-J. Huang

Appendix

Materials and Methods

Embryoid body (EB) formation and in vitro spontaneous differentiation

Based on our previous work (Yan et al. 2010), iPSC colonies were harvested from feeders by treatment with 0.2 - 0.5 mg/ml dispase for 20-30 min until complete detachment. The collected colonies were then transferred to wells of 6-well plates (low attachment) containing embryonic stem cell (ESC) media without bFGF. The media were changed every other day to allow EB formation as floating culture. After 8 days, EBs were transferred to gelatin-coated plates to allow attachment and cultured in the same medium for another 8 days during which time cells migrated out from attached EBs. Cell cultures were then fixed for immunocytofluorescence analysis.

Immunocytofluorescence

Cell cultures were stained based on methods we previously reported (El Ayachi et al. 2018; Zou et al. 2012). Briefly, cultures were fixed in 100% ice cold methanol, followed by treatment with blocking buffer (32.5 mM NaCl, 3.3 mM Na_2HPO_4, 0.76 mM KH_2PO_4, 1.9 mM NaN_3, 0.1% (w/v) bovine serum albumin (BSA), 0.2% (v/v) Triton-X 100, 0.05% (v/v) Tween 20 and 5% goat serum) for 30 min. Primary antibodies were then added and cultures placed at 4^{o}C overnight. For secondary antibody treatment, cultures were incubated with anti-mouse, or anti-rabbit antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, www.invitrogen.com/site/us/en/home.html) for 1 h at room temperature and the cell nuclei stained with DAPI (Invitrogen). Images were analyzed with a

fluorescence microscope. **Supplemental Table S1** contains information of all antibodies used for the staining.

Teratomas formation

The method followed our previous reports (El Ayachi et al. 2018; Yan et al. 2010; Zou et al. 2012). Briefly, SCAP-O iPSCs and TF-SCAP-O iPSCs ~70% confluent in a 6-well plate were harvested by collagenase IV treatment, collected and suspended in the DMEM/F12 and Matrigel (3:1) solution. The cells were injected intramuscularly into the right and/or left hind legs of a SCID mouse (SCID, NOD.CB17-Prkdc-scid/J; Jackson Laboratory, Bar Harbor, ME). After 7-9 weeks, formed tumors were dissected and fixed with PBS containing 4% paraformaldehyde. Paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin.

Lineage differentiation

i) Osteogenic differentiation

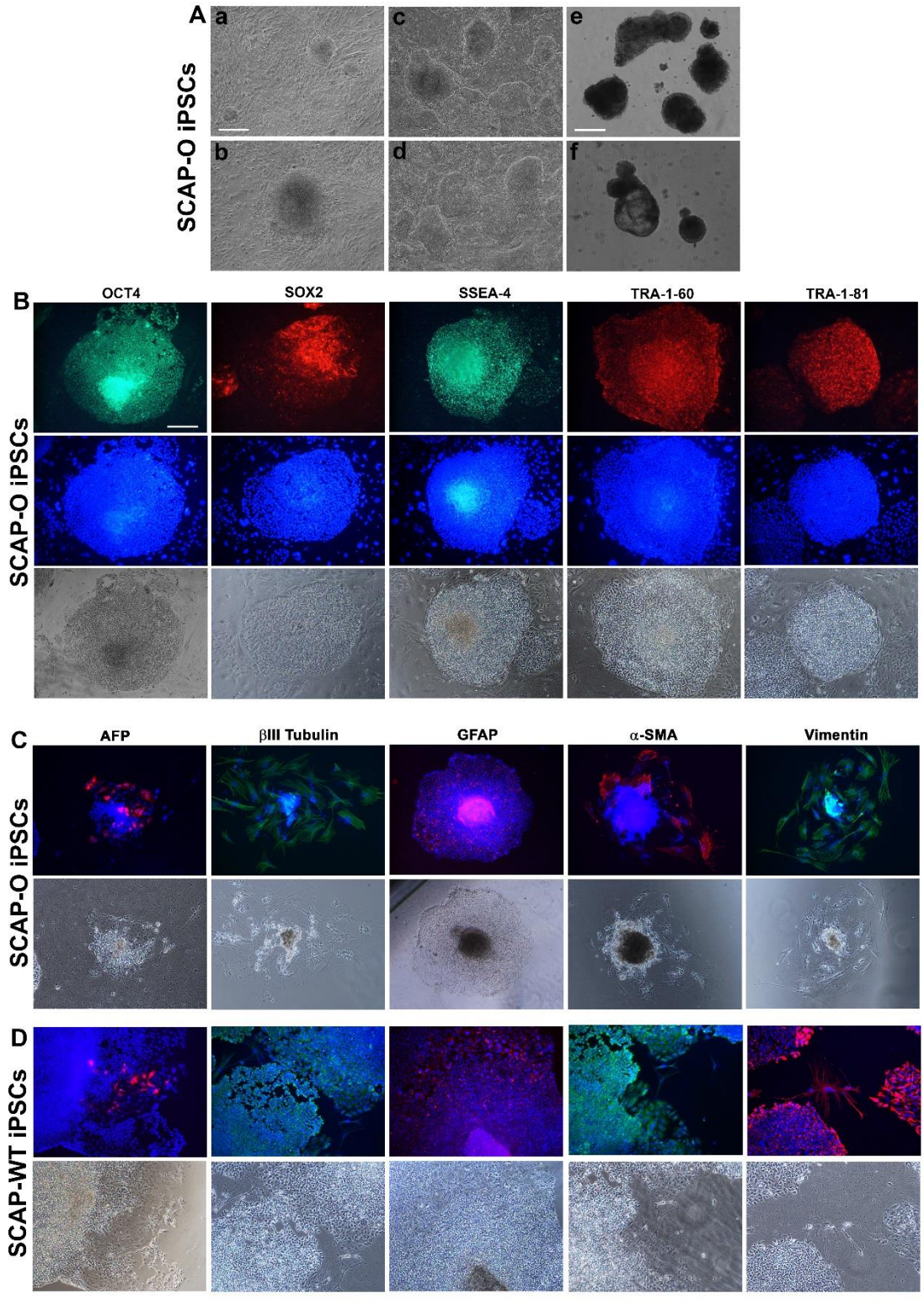
TF-iPSCs were first guided to become mesenchymal stem cells before osteogenic stimulation based a previous report (Zou et al. 2013). iPSCs were passaged and after 3 days the medium was replaced with an MSC medium containing DMEM-low glucose, 10% FBS, 2 mM L-Glutamine and 1% pen/streptomycin. The MSC medium was changed every 2 days for 14 days. Intermediate phase of differentiating iPSCs emerged and passaged onto 0.1% gelatin-coated dishes for further culturing and expansion for 3 passages (at a 1:3 ratio). A morphologically homogeneous population of fibroblast-like cells became evident and were seeded into 24-well plates. Osteogenic stimulation began at subconfluence (70-80%) in a medium containing 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/ml ascorbate phosphate, 10 nM 1, 25 dihydroxyvitamin D_3 and 10% FBS for 21 days and the

medium was changed every 3 days. Cultures were harvested for qRT-PCR and sequencing analysis, or fixed in 60% isopropanol, and mineralization of extracellular matrix stained with 1% Alizarin red S (ARS) (Huang et al. 2010; Yu et al. 2015).

ii) Neurogenic differentiation in vitro

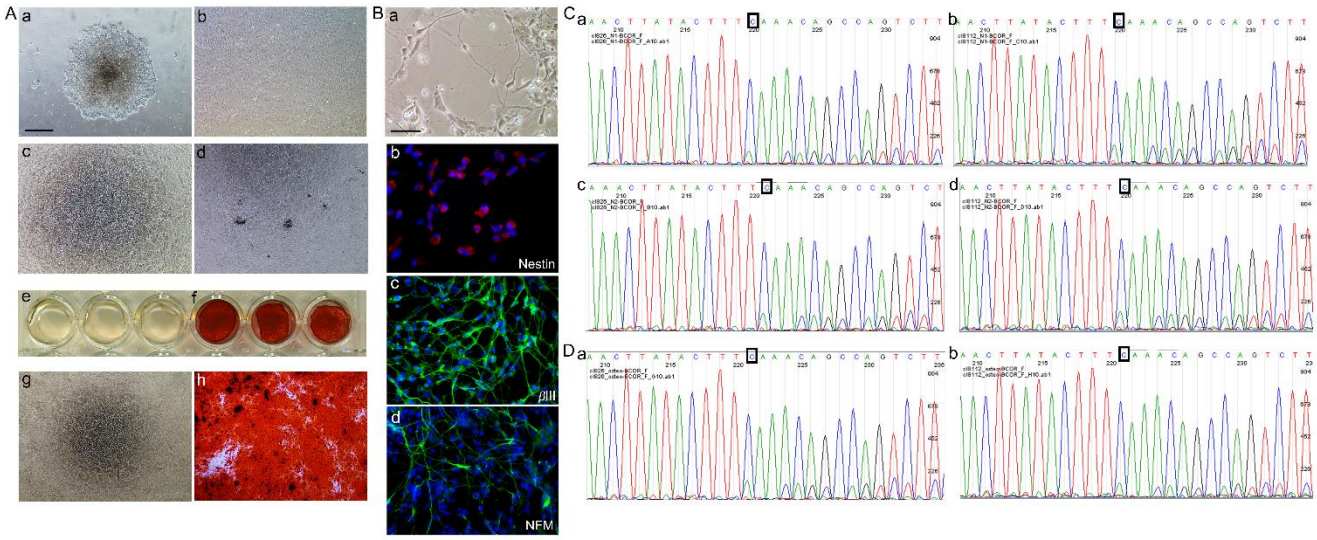
Direct induction of neurogenesis

This method followed what we previously reported, with modifications (El Ayachi et al. 2018). In brief, TF-iPSC colonies grown on MEFs were passaged onto Geltrex matrix feeder-free (Invitrogen) coated-wells of six wells plates as small clumps at 2.5×10^5 cells per well and cultivated with TESR medium. When colonies reached 70–80% confluency, the medium is replaced with a neural induction medium (NIM, containing Neurobasal Medium and Neural Induction Supplement, GIBCO® PSC Neural Induction Medium, ThermoFisher Scientific) for 7 days and medium was changed daily to derive neural stem/progenitor cells (NSCs). Subsequently, the colonies are dissociated with Stem pro Accutase (ThermoFisher Scientific) and the NSCs harvested again onto Geltrex coated wells. Cells were grown in neural expansion medium (NEM, containing Neurobasal Medium, Advanced DMEM/F12, Neural Induction Supplement, ThermoFisher) for expansion and passaged several times. At each passaging, cells were treated with 10 μM ROCK inhibitor to prevent cell death. NIM-NSCs were then seeded onto 1% polylisine-coated wells and incubated in neural differentiation medium (NDM, containing Neurobasal medium, SHH 100 ng/ml. RA 100 nM) for ~3 weeks and then changed to neuronal differentiation medium (contained Neurobasal medium, SHH 50ng/mL, RA 50 nM, BDNF 10 ng/ml, GDNF 10 ng/ml, IGF1 10 ng/ml, cAMP 1 μM, Ascorbic Acid 200 ng/ml) for ~2 weeks.


A
SCAP-O iPSCs
a
c
e
b
d
f
B
OCT4
SOX2
SSEA-4
TRA-1-60
TRA-1-81
SCAP-O iPSCs
C
AFP
βIII Tubulin
GFAP
α-SMA
Vimentin
SCAP-O iPSCs
D
SCAP-WT iPSCs

Supp Fig 1

Supplemental Figure 1. Reprogramming of SCAP-O into iPSCs. **(A)** Lentiviral vector set pSin-EF2-gene-Pur was used to reprogram SCAP-O. **(Aa,b,)** Emergence of ES cell-like colonies at passage 0. **(Ac,d)** At passage 3 after expansion; **(Ae,f)** EB formation after 1 wk. **(B)** Immunocytofluorescence of ES cell-associated gene expression by SCAP-O iPSC clones. Top images: gene fluorescence staining; middle images: DAPI nuclear stain; bottom images: bright field. **(C)** Immunocytofluorescence of marker expression after EB-mediated spontaneous differentiation. SCAP-WT were the control cells. AFP (endoderm), βIII tubulin and GFAP (ectoderm), α-SMA (mesoderm), and vimentin (mesoderm and parietal endoderm) representing three germ layers. Scale bars: (Aa-d) 500 μm; (Ae,f; B; C) 100 μm.



Supp Fig 2

Supplemental Figure 2. Osteo-/Neuro-differentiation of TF SCAP-O$^{BCOR\text{-mut}}$ iPSCs and sequence analysis of *BCOR* mRNA after differentiation **(A)** Osteo-differentiation. **(Aa)** Cells before differentiation. iPSCs grown on Matrigel/TESR2. **(Ab)** Cell cultures were replaced with MSC medium and passaged 3 times showing fibroblast morphology. **(Ac,d)** After 14 days in MSC medium, cells were passaged to 24-well plates, grown to 70-80% confluent and switched with osteogenic (Os) differentiation medium for one week. **(Ac)** Control culture without Os medium showing cells became confluent with fibroblast-like morphology. **(Ad)** Os group showing initial deposition of mineral nodules in cultures. **(Ae,f)** After 3 weeks of Os stimulation, cultures were stained with Alizarin Red S. **(Ae)** Left 3 wells are controls. **(Af)** Right 3 wells are Os group. **(Ag,h)** Higher magnification of Alizarin Red S stain of cultures. **(Ag)** Control. **(Af)** Os group. Scale bars: 500 um, images in (Aa-d, Ag,h). **(B)** Neural differentiation for 5 weeks. **(Ba)** Bright field image of neuron-like cells and staining of neural markers nestin **(Bb)**, βIII-tubulin **(Bc)** and NFM **(Bd)**. Scale bar: 50 um all images in (B). **(C, D)** Sequence analysis of *BCOR* mRNA expressed in differentiated SCAP-O$^{BCOR\text{-mut}}$ iPSCs. Two representative clones are shown. **(Ca,b)** Neuro-differentiation at 3 weeks. **(Cc,d)** Neuro-differentiation

at 5 weeks. **(Da,b)** Osteo-differentiation from iPSCs to MSCs (2 weeks) and osteogenic induction (3 weeks) total of 5 weeks (sequence performed at 5 weeks).

Supplemental Table S1: Antibodies used for immunocytostaining

Antibody	Company	Original concentration	Dilution
Primary antibody			
OCT4 (mouse anti-human)	Santa Cruz Biotech., Inc	0.2 mg/ml	1:400
SOX2 (rabbit anti-human)	Santa Cruz Biotech., Inc	0.2 mg/ml	1:100
SSEA-4 (mouse anti-human)	Chemicon Int Inc.,	1 mg/ml	1:400
TRA-1-60 (mouse anti-human)	Chemicon Int Inc.,	1 mg/ml	1:400
TRA-1-81 (mouse anti-human)	Chemicon Int Inc.,	1 mg/ml	1:400
βIII-tubulin (mouse anti-human)	Sigma-Aldrich	1.0 mg/ml	1:200
GFAP (mouse anti-human)	Sigma-Aldrich	7.9 mg/ml	1:400
CNPase (Mouse anti-human)	Millipore	1mg/ml	1:200
Nestin (mouse anti-human)	R & D	500 µg/ml	1:200
NFM	Invitrogen	0.5 mg/ml	1:200

(mouse anti-human)			
AFP (α 1 fetoprotein) (rabbit anti-human)	Abcam Inc.	2 mg/ml	1:100
αSMA (α smooth muscle actin) (rabbit anti-human)	Abcam Inc.	0.2 mg/ml	1:200
Vimentin (mouse anti-human)	Abcam Inc.	1 mg/ml	1:20
Secondary antibody			
Goat anti-mouse Alexa Fluor 488	Life technologies	2 mg/ml	1:500
Goat anti-mouse Alexa Fluor 594	Life technologies Invitrogen	2 mg/ml	1:500
Donkey anti-rabbit Alexa Fluor 594	Life technologies	2 mg/ml	1:500

Reference

El Ayachi I, Zhang J, Zou XY, Li D, Yu Z, Wei W, O'Connell KMS, Huang GT. 2018. Human dental stem cell derived transgene-free ipscs generate functional neurons via embryoid body-mediated and direct induction methods. J Tissue Eng Regen Med. 12(4):e1836-e1851.

Huang GT-J, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, Shi S. 2010. Stem/progenitor cell–mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Tissue Engineering Part A. 16(2):605-615.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. 2010. Ips cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells and Development. 19(4):469-480.

Yu Z, Gauthier P, Tran QT, El Ayachi I, Bhatti F-U-R, Bahabri R, Al-Habib M, Huang GTJ. 2015. Differential properties of human alp+ periodontal ligament stem cells vs their alp- counterparts. Journal of Stem Cell Research and Therapy. 5(7):292.

Zou L, Luo Y, Chen M, Wang G, Ding M, Petersen CC, Kang R, Dagnaes-Hansen F, Zeng Y, Lv N et al. 2013. A simple method for deriving functional mscs and applied for osteogenesis in 3d scaffolds. Sci Rep. 3:2243.

Zou XY, Yang HY, Yu Z, Tan XB, Yan X, Huang GT. 2012. Establishment of transgene-free induced pluripotent stem cells reprogrammed from human stem cells of apical papilla for neural differentiation. Stem Cell Res Ther. 3(5):43.