

FGF signaling mediates definitive endoderm formation by regulating epithelial-to-mesenchymal transition and cell proliferation

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ABSTRACT FGF signaling pathway is imperative for definitive endoderm (DE) differentiation from human embryonic stem cells (hESCs), which always accompanies an epithelial-to-mesenchymal transition (EMT) process. However, whether there is an association between FGF signaling and the EMT during DE formation *in vitro* has remained elusive. In the present study, we identify that several FGF family members were significantly activated during the differentiation of hESCs toward DE. Inhibition of FGF signaling by an efficient and selective inhibitor BGJ398 abolishes both the EMT and DE induction by blocking the activation of the zinc-finger transcription factor SNAI1 which is a direct transcriptional repressor of cell adhesion protein CDH1. In addition, cell proliferation is also severely influenced by attenuating the FGF signaling. Collectively, we propose that the FGF signaling promotes the DE formation through mediating the EMT and cell proliferation.


KEY WORDS: *definitive endoderm, FGF signaling, EMT, cell proliferation*

Introduction

Fibroblast growth factor (FGF) signaling pathway, activated by a ligand-receptor interaction, controls a multitude of developmental processes such as cell proliferation, migration and differentiation during the mammalian development. The FGF family comprises of 18 ligands and 4 receptors (*FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*), and they exhibit specific expression profiles at the distinct stages throughout embryonic development (Deng *et al.*, 1994 a; Yamaguchi and Rossant 1995). FGF signaling is known to be essential for mouse gastrulation, during which epiblast cells undergo an epithelial-to-mesenchymal transition (EMT) and then give rise to the mesodermal and definitive endodermal germ layers. Targeted disruption of FGF receptor 1 in mice led to downregulation of CDH1 and inhibition of the EMT process in epiblast cells at the primitive streak, and *Fgfr1*^{-/-} cells hardly gave rise to the mesoderm lineage (Ciruna and Rossant 2001 b). In addition, FGFR1 signaling was also demonstrated to be involved in mesodermal cell migration out of the primitive streak during mouse gastrulation (Yamaguchi *et al.*, 1994). Although activation of FGF signaling is previously

considered to be indispensable for generation and patterning of mesoderm rather than endoderm during embryogenesis (Bottcher and Niehrs 2005), accumulating evidences indicate that except TGF- β , WNT and BMP signals, FGF signaling is also involved in efficient definitive endoderm (DE) specification from mouse ESCs (Gadue *et al.*, 2005; Morrison *et al.*, 2008). TGF- β /nodal/activin signaling plays an important role in the segregation of mesoderm and endoderm from primitive streak during both the vertebrate early embryo development and pluripotent mammalian stem cells differentiation, and other signaling pathways, particularly FGF and WNT, are also demonstrated to be imperative for the specification of primitive streak and the induction of endodermal markers during mouse embryogenesis (Tam and Loebel 2007). Blocking FGFR1 signaling by a chemical inhibitor during the differentiation of mouse ESCs resulted in diminished generation of the endoderm lineage and downregulation of multiple related regulatory genes (Morrison

Abbreviations used in this paper: DE, definitive endoderm; EMT, epithelial-to-mesenchymal transition; FGF, fibroblast growth factor; hESC, human embryonic stem cell; RT-qPCR, quantitative reverse-transcription polymerase chain reaction.

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et al., 2008). Genetic disruption of *Fgf8* in mouse embryo developed few mesoderm- or endoderm-derived tissues (Sun et al., 1999).

The process of epithelial-to-mesenchymal transition, characterized with upregulation of EMT-related transcription factors such as *SNAI1* and cell migration, is inherent to the process of human DE specification *in vitro*. We have previously reported that genetic ablation of *SNAI1* in hESCs disturbed the EMT process which was considered to be mainly mediated by autocrine TGF- β signaling, and severely inhibited the induction of endodermal markers such as *SOX17* and *FOXA2* (Li et al., 2017). FGF signals were reported to efficiently drive the DE differentiation from human pluripotent stem cells (Bernardo et al., 2011; Loh et al., 2014). Yet, whether FGF signaling is involved in Activin A-induced EMT events and DE formation *in vitro* remain unclear, though FGF signaling was ever reported to upregulate the zinc-finger transcription factor *SNAI1* (a repressor of cell adhesion protein *CDH1*) and promote migration activity in epithelial tumor cells (Battle et al., 2000). In addition, FGFs also contributed to the proliferation and movements of endothelial cells during wound healing (Folkman and Klagsbrun 1987). In this present study, we identify that the activation of FGF signaling is responsible for part of Activin A-induced EMT process via upregulating expression of *SNAI1*, downregulating expression of *CDH1* and stimulating cell proliferation. Pharmacological inhibition of FGF signaling by an effective and selective inhibitor blocks the EMT process, impairs cell proliferation, and consequently attenuates DE formation. Our results indicate that FGF signaling pathway participates in mediating the Activin-induced EMT process and endodermal lineage specification from human embryonic stem cells.

Results

FGF signaling is activated during the definitive endoderm formation and its inhibition leads to the failure of endoderm induction

As it was shown in Figure 1A and 1B, DE cells were induced successfully using a serum-free, chemically defined protocol we described before (Li et al., 2017). Primitive streak specific genes like *EOMES*,

MIXL1, and *WNT3* were dramatically upregulated, as well as DE markers such as *SOX17*, *FOXA2* and *GSC* (Fig. 1C). To investigate whether the FGF signaling is involved in Activin A-induced DE differentiation, we examined the mRNA expression of FGF family members. Expression of *FGFR2*, *FGFR3* and *FGFR4* was observed to increase significantly, accompanied with remarkable upregulation of *FGF17* and *FGF8*. The classical primitive streak marker *FGF4* remained the same expression level as hESCs (Fig. 1D), which is in accordance with others' report (D'Amour et al., 2005). In order to make it clear whether signaling downstream of FGFR was required for the differentiation of ESCs to DE *in vitro*, we pharmacologically inhibited this signaling using an effective and selective FGF receptor inhibitor, BGJ398. As shown in Fig. 2A, the expression of *FGFR2/3/4*, *FGF17* and *FGF8* was observed to be remarkably inhibited by BGJ398. The induction of mesendoderm and endoderm makers such as *SOX17*, *FOXA2* and *WNT3* was significantly disturbed by BGJ398 (Fig. 2B). As it was shown in

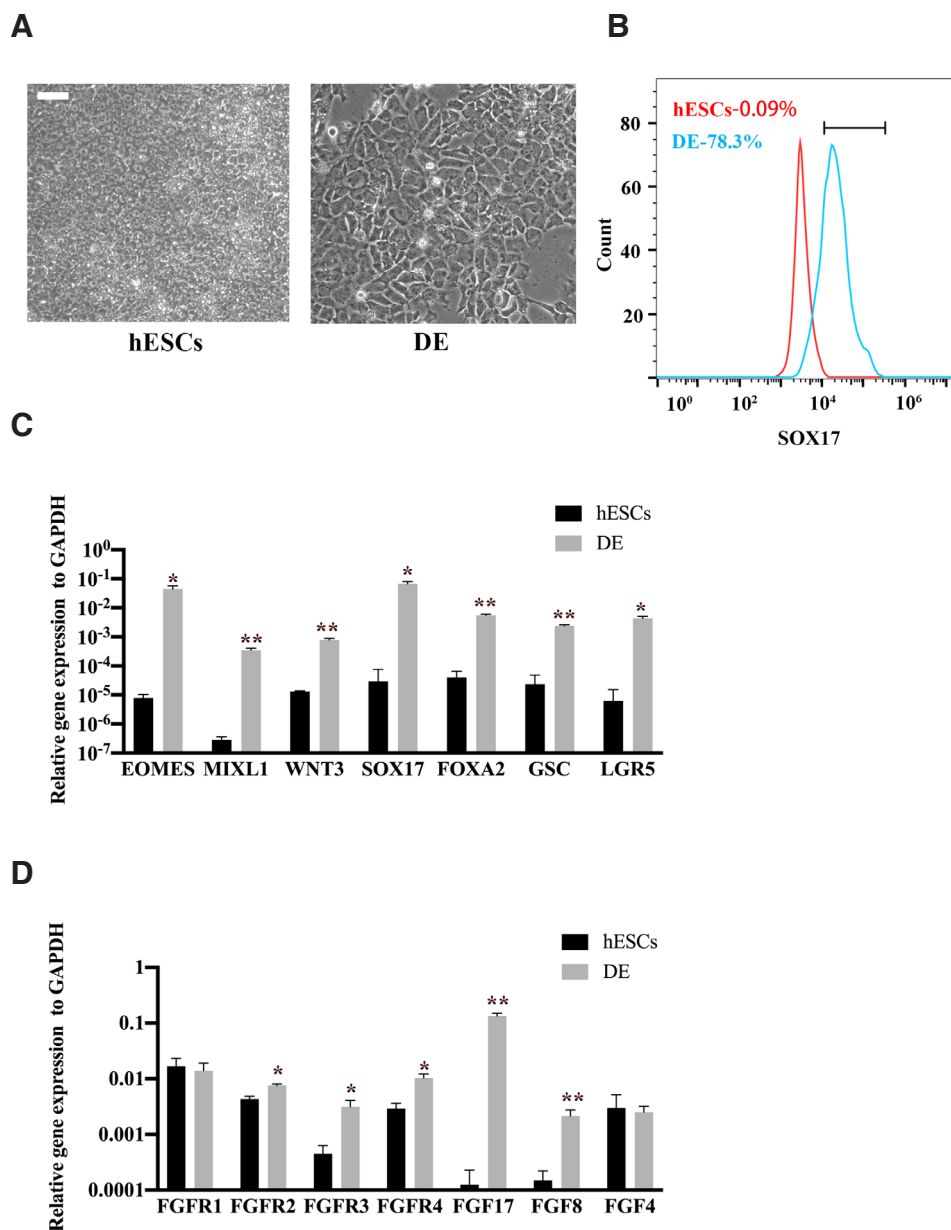


Fig. 1. Gene expression analysis of definitive endoderm differentiation from hESCs. (A) Representative live photos of undifferentiated hESCs and Activin A-induced cells are shown. The scale bar indicates 20 μ m (B) Flow cytometry analysis of undifferentiated hESCs and Activin A-induced cells. (C, D) RT-qPCR analysis for the indicated genes expressed in undifferentiated hESCs and Activin A-induced cells. Shown are the means \pm s.d. of three replicates. * p < 0.05; ** p < 0.01, compared to hESCs.

Supplementary Fig. S1, the results above were also confirmed by using two different human iPSCs lines (hiPSCs-1 originated from urine and hiPSCs-2 derived from skin fibroblast).

An EMT during definitive endoderm formation is inhibited by BGJ398

We and others have previously reported that an EMT process with obvious changes in the expression of specific genes and cell migration capacity accompanied definitive endoderm differentiation from hESCs *in vitro* (D'Amour *et al.*, 2005; Li *et al.*, 2017). As shown in Fig. 3A, SOX17-positive cells were characterized with diminished expression of epithelial marker CDH1 and the induction of mesenchymal markers such as SNAI1 and CDH2, compared to the original hESCs. Furthermore, the formation of stress fibers inside the cells, which is a characteristic of mesenchymal cells, was very evident during DE differentiation. To determine whether the endogenously activated FGF signaling pathway is involved in the EMT process during DE formation, we blocked this signaling by BGJ398 and detected its effect on EMT by immunofluorescence staining. We observed that few SOX17-positive cells were generated in the presence of BGJ398. The remaining cells after BGJ398 treatment maintained the expression of epithelial marker CDH1, and the activation of SNAI1 in the nucleus was hardly detected. Although mesenchymal marker CDH2 was also observed in some BGJ398-treated cells, most cells expressed very finite CDH2 protein in the cell membrane compared to DE cells. The F-actin staining in BGJ398-treated cells resembled that of undifferentiated hESCs, rather than DE cells. We then measured the mRNA and protein expression of several EMT related markers during the

endodermal differentiation (Fig. 3B and Fig. 3C). In the absence of BGJ398, the mesenchymal markers such as CDH2 and SNAI1 were upregulated and the epithelial marker CDH1 was downregulated mildly. In the presence of BGJ398, the activation of mesenchymal markers like CDH2 and SNAI1 was blocked severely. The epithelial marker CDH1, however, failed to downregulate. Overall, the inherent EMT programme (activation of SNAI1, downregulation of CDH1) in DE formation was drastically inhibited by the FGF receptor inhibitor BGJ398.

Cell proliferation is severely disturbed and cell fate changes toward neuroectoderm

It is obvious that cell number dropped very rapidly after the

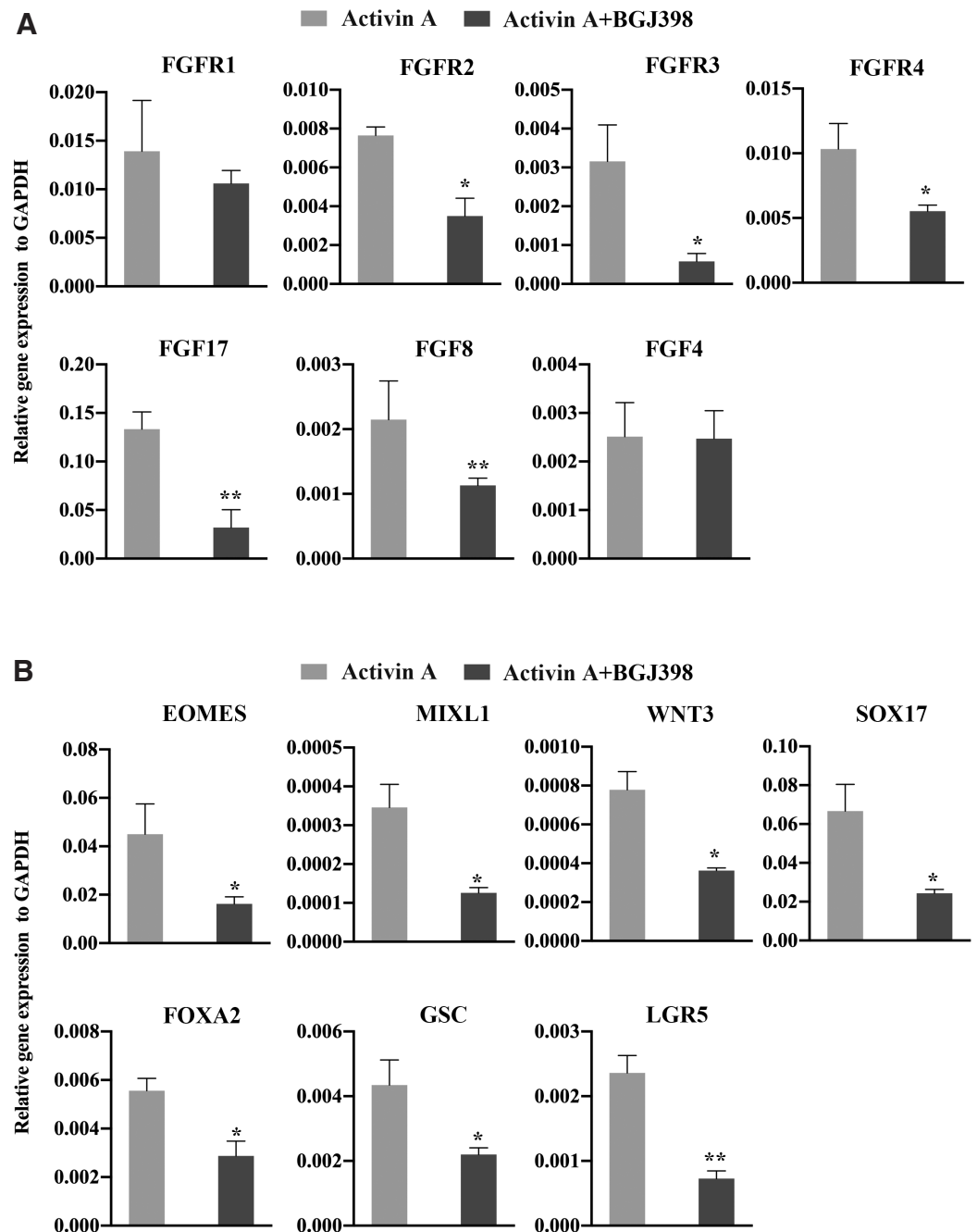


Fig. 2. Definitive endoderm induction is disturbed by BGJ398, as well as gene expression of FGF family members. (A,B) RT-qPCR analysis for the indicated genes expressed in Activin A-induced cells in the absence and presence of BGJ398. Data represent means \pm s.d. from three biological repeats. * $p < 0.05$; ** $p < 0.01$, compared to Activin A-induced cells in the absence of BGJ398.

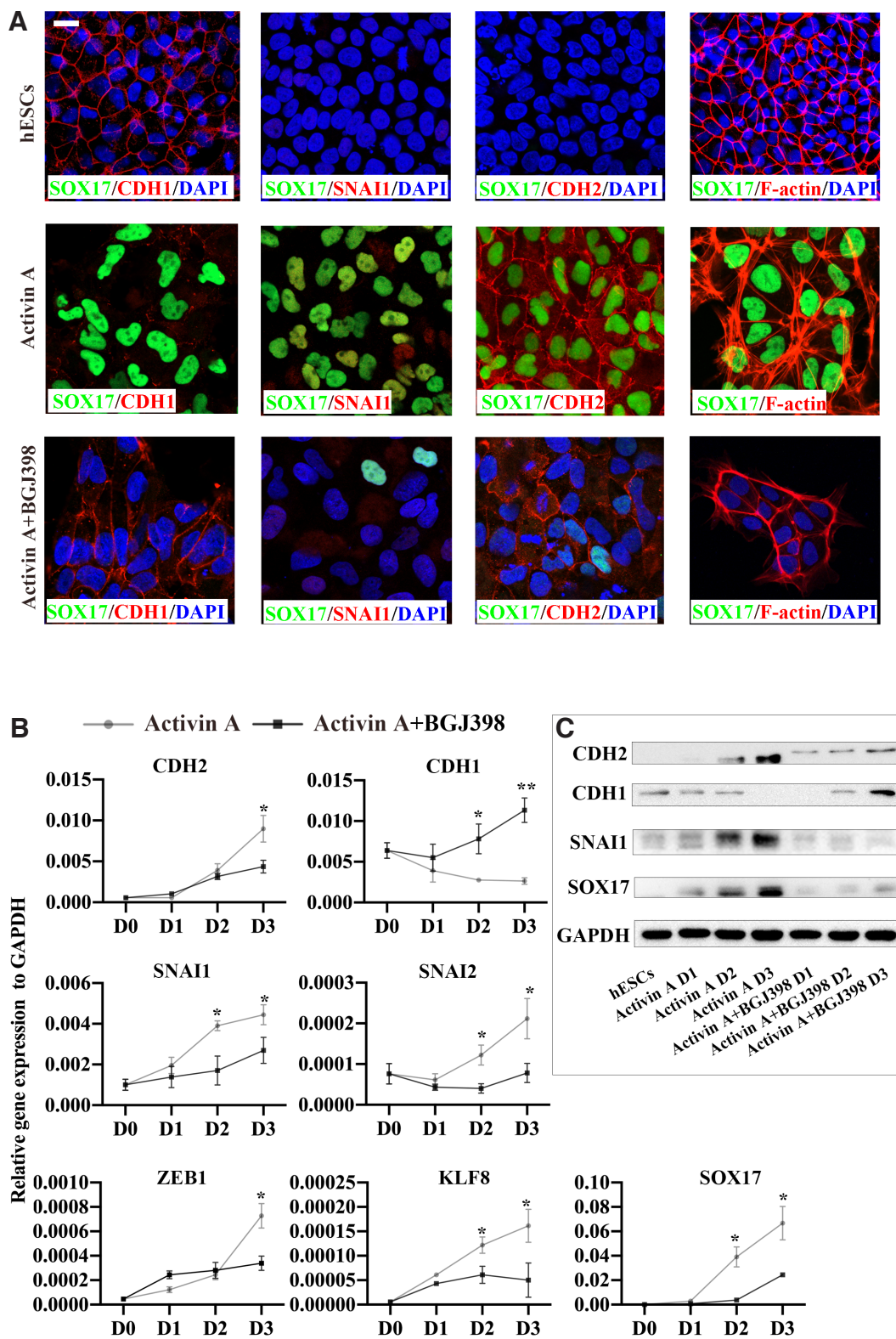


Fig. 3. An epithelial-to-mesenchymal transition (EMT) during definitive endoderm differentiation is blocked by BGJ398. **(A)** Immunofluorescence images of undifferentiated hESCs and Activin A-induced cells with or without BGJ398 treatment. The scale bar indicates 20 μ m. **(B)** Time-course RT-qPCR analysis for EMT associated genes expressed in hESCs and Activin A-induced cells with or without BGJ398 treatment. Shown are the means \pm s.d. of three replicates. * p <0.05; ** p <0.01, compared to Activin A-induced cells in the absence of BGJ398. **(C)** Western blot analysis for EMT and DE associated markers at day 0, day 1, day 2 and day 3.

inhibitor BGJ398 treatment during Activin A-induced DE formation (Fig. 4A). Cell proliferation curve was drawn during DE differentiation in the absence or presence of BGJ398 (Fig. 4A). At day 1, the number of living cells dropped rapidly in comparison with day 0 whether in the absence or presence of the FGF receptor inhibitor. Cell number increased gradually from day 1 to day 3, however, cell number continued to decrease in the presence of BGJ398. Since DE formation was seriously inhibited by the inhibitor BGJ398, we then examined the expression of other cell lineage markers in order to determine the direction of cell differentiation (Fig. 4B). The ectoderm markers such as *PAX6*, *SOX2* and *NESTIN*, rather than mesoderm markers like *MESP1*, *MESP2* and *HAND1* (data not shown), were activated in Activin A-induced cells treated with BGJ398 during the DE differentiation of hESCs. As shown in Figure 4C, similar results were obtained with a second FGF inhibitor AZD4547. AZD4547 treatment led to the inhibition of FGF family members and DE markers expression, and the cells were converted toward the neuroectoderm lineage.

Discussion

Previously, the FGF signaling pathway is usually regarded as a mediator of morphogenetic movement and participates in the migration and patterning of mesoderm at gastrulation. Recent

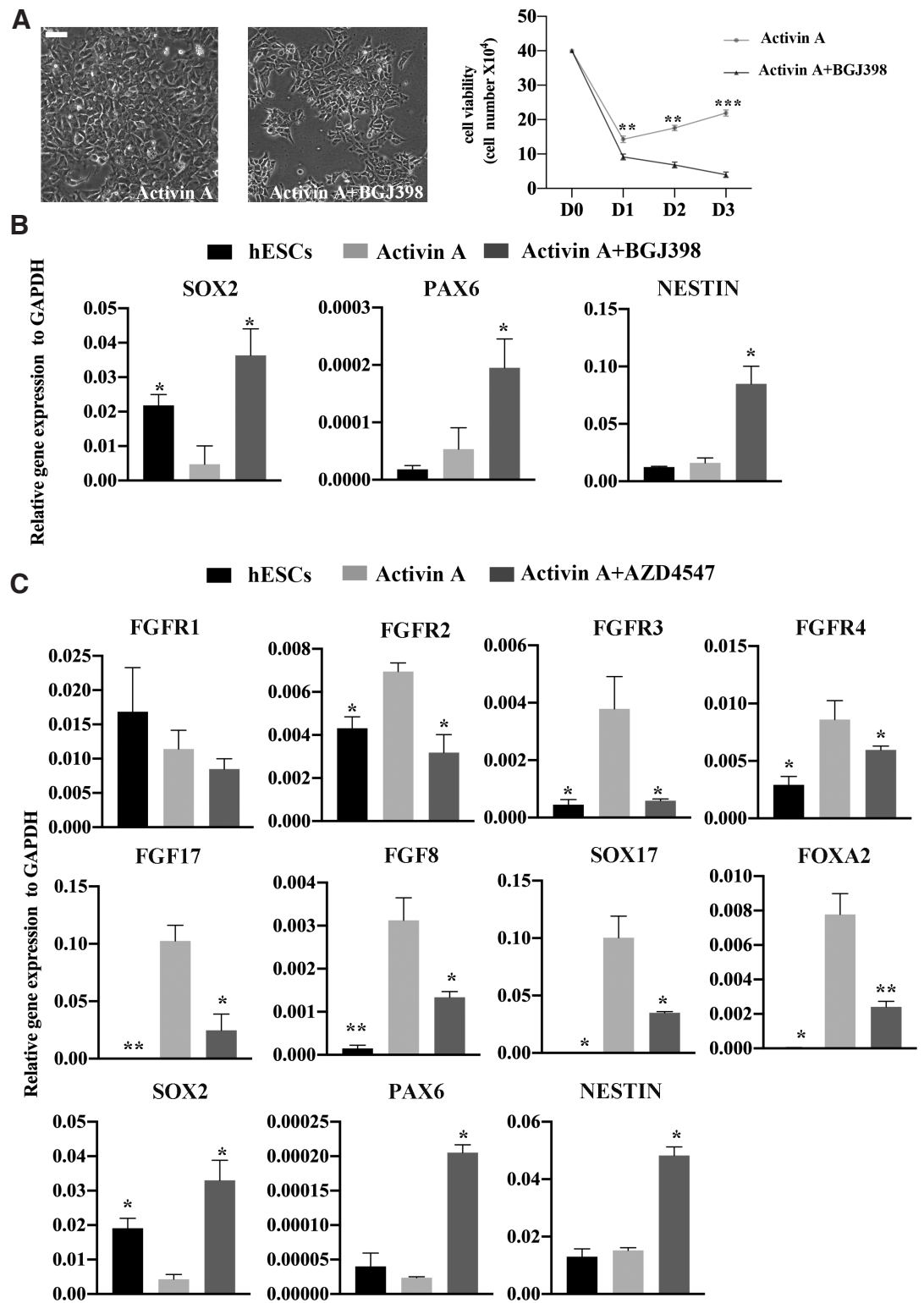


Fig. 4. Cell proliferation is disturbed by BGJ398 and cell fate changes to neuroectoderm. (A) Phase contrast images of Activin A-induced cells treated with or without BGJ398. The scale bar indicates 20 μ m. Cell proliferation curve was drawn by counting living cell numbers, and data represent means \pm s.d. of three replicates. ** p <0.01, *** p <0.001, compared to Activin A-induced cells in the absence of BGJ398. (B) RT-qPCR analysis for ectodermal markers expressed in hESCs and Activin A-induced cells with or without BGJ398 treatment. Data represent means \pm s.d. of three replicates. * p <0.05, compared to Activin A-induced cells in the absence of BGJ398. (C) RT-qPCR analysis for the indicated genes expressed in hESCs and Activin A-induced cells in the absence and presence of AZD4547. Data represent means \pm s.d. of three replicates. * p <0.05; ** p <0.01, compared to Activin A-induced cells in the absence of AZD4547.

evidences indicates that FGF signaling also plays a pivotal role in directing hESCs toward endoderm (Ang *et al.*, 2018; Loh *et al.*, 2014; Sui *et al.*, 2012; Yiangou *et al.*, 2018). Specifically, FGF2 and FGF4 have been demonstrated to be essential in Activin A-induced DE formation (Loh *et al.*, 2014; Sui *et al.*, 2012), and a single FGFR1 inhibitor treatment during the entire *in vitro* differentiation procedure resulted in the failure of anterior DE generation (Morrison *et al.*, 2008).

We have ever reported that TGF- β /nodal/activin signaling is imperative for DE differentiation *in vitro*, and its pharmacological inhibition by Repsox blocks DE formation (Li *et al.*, 2017). However, whether the FGF signaling pathway participates together with TGF- β signal to acquire DE from hESCs remains elusive. In this present study, we discovered that FGF signaling was remarkably activated during the differentiation of hESCs toward DE, although no exogenous FGF ligands were added. The endogenous expression of *FGF17* and *FGF8* was increased largely during the DE formation, which is in agreement with others' report (Cheng *et al.*, 2012). *FGF8* and *FGF17* were reported to belong to one of the FGF subfamilies, and they have very similar biochemical functions in developmental biology, cell proliferation and migration. They both have very strong interaction with *FGFR3* and *FGFR4*, moderate interaction with *FGFR2*, and minimal interaction with *FGFR1* (Xu *et al.*, 2000).

FGF signaling was demonstrated to involve in DE specification from mouse ESCs (Gadue *et al.*, 2005; Morrison *et al.*, 2008). The blocking of FGF signaling in mice led to diminished endoderm lineage generation and few endoderm-derived tissues (Sun *et al.*, 1999; Morrison *et al.*, 2008). In addition, targeted disruption of FGF signaling was also found to block the EMT process at the primitive streak before endoderm and mesoderm generation (Ciruna and Rossant 2001 b). We and others have observed synchronous EMT events during DE formation derived from hESCs *in vitro* (D'Amour *et al.*, 2005; Li *et al.*, 2017). Under the Activin-induced DE protocol, we found that FGF signaling was activated during the DE generation. To make it clear whether there is a close relationship between this inherent EMT process and FGF signaling, we detected the expression of EMT and DE-related markers in the presence of a selective inhibitor of the FGF receptor (BGJ398) in endoderm differentiation. Addition of BGJ398 resulted in decreased mRNA expression of FGF ligands (*FGF17* and *FGF8*) and receptors (*FGFR2*, *FGFR3* and *FGFR4*), which were imperative to the activation of FGF signaling. Inhibition of FGF signaling then disrupted the upregulation of EMT-related transcription factors such as *SNAI1*, which was reported to be responsible for the occurrence of EMT events and DE formation *in vitro* (Li *et al.*, 2017). In addition, other EMT events including the switch between cell adhesion protein, and stress fibers formation were also disturbed by BGJ398. These results above agreed with others' report in mouse that FGFR1 signaling regulated EMT process and mesoderm formation by controlling *SNAI1* and *CDH1* expression (Ciruna and Rossant 2001a). In addition, these BGJ398-treated cells exhibited a noticeable proliferative defect compared to normal DE cells, indicating that FGF signaling played an important role in regulating cell growth.

It was reported that inhibition of FGFR1 signaling using a selective inhibitor PD173074 after the primitive streak formation would lead to the mesoderm generation (Loh *et al.*, 2014), while few *Fgfr1* *-/-* cells contributed to mesoderm and endodermal lin-

eages (Ciruna *et al.*, 1997; Deng *et al.*, 1994 b). To make it clear into which cell lineage these BGJ398-treated cells differentiated, we examined the expression of ectodermal and mesodermal genes (data not shown). We found that some ectoderm markers such as *PAX6*, *SOX2* and *NESTIN* upregulated significantly, while the mesoderm makers like *MESP1*, *MESP2* and *HAND1* failed to be activated. The results indicated that inhibition of FGF signaling during the whole DE induction process preferred the neuroectoderm lineage. Taken together, in this present study, we discovered that FGF signaling mediated the definitive endoderm differentiation from hESCs predominantly through controlling EMT and cell proliferation. Inhibition of FGF signaling by a selective FGFR inhibitor could disturb the inherent EMT process and give rise to few DE cells.

Materials and Methods

Cell culture and differentiation

Undifferentiated human H1 embryonic stem cells (WiCell) and two different human iPSCs cell lines without any mycoplasma contamination were routinely cultured on Matrigel (BD Biosciences, USA) coated plates in mTeSR1 medium (STEMCELL Technologies, Canada). hESCs were manually passaged at 1:6 to 1:8 using Accutase (Sigma, USA) every 3-4 days. Definitive endoderm differentiation was carried out for three days in RPMI 1640/B27 minus insulin medium (ThermoFisher Scientific, USA) supplemented with 100 ng/mL Activin A (Peprotech, USA) and other supplements as previously described (Li *et al.*, 2017). To blocking the FGF signaling pathway in DE differentiation, an effective and selective FGFR1/2/3 inhibitor BGJ398 (50 nM, Selleck Chemicals, USA) or AZD4547 (50 nM, Selleck Chemicals, USA) was added during the whole induction process.

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol (ThermoFisher Scientific, USA), and 2 mg of total RNA was prepared for reverse-transcription with Rever-Trace (TOYOBO, Japan). The cDNA products were then diluted and used as PCR templates. The PCR reactions were performed using a SYBR® Premix Ex Taq™ Kit (Takara Bio, Japan) on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Expression of target genes was normalized to *GAPDH* expression level. Primer sequences for RT-qPCR were listed in the Supplementary Data 1. The experiments were carried out in triplicate.

Immunofluorescence staining

Differentiated cells were fixed with 4% wt/vol paraformaldehyde at 25 °C for 30 min; then cells were blocked and permeabilized using the blocking solution containing 10% fetal bovine serum and 0.3% Triton X-100 for 30 min. Cells were incubated with a primary antibody diluting in the blocking solution at 4 °C overnight. A secondary antibody was added to incubate the fixed cells at room temperature for 1 h. Then cells were stained with DAPI (Sigma, USA) for 5 min and photographed by Zeiss LSM 710 confocal microscope (Carl Zeiss, USA). The primary and secondary antibodies used were listed below: goat anti-SOX17 (R&D systems, USA); rabbit anti-CDH1 (ABclonal Biotechnology, China); mouse anti-CDH2 (BD Bioscience, USA); mouse anti-SNAI1 antibody (Cell signaling technology, USA); Alexa Fluor® 568 donkey anti-rabbit IgG (Invitrogen, USA); Alexa Fluor® 568 donkey anti-mouse IgG (Invitrogen, USA); Alexa Fluor® 488 donkey anti-goat IgG (Invitrogen, USA). For F-actin staining, cells were incubated with 1 unit/mL rhodamine phalloidin (Invitrogen, USA) for 1 h at room temperature.

Flow cytometry analysis

Targeted cells were fixed in 4% paraformaldehyde for 15 min and then permeabilized in 0.1% wt/vol Triton-X 100 for 15 min. Cells were then incu-

bated with Alexa Fluor® 488 mouse anti-human SOX17 (BD Pharmingen, USA) in 2% fetal bovine serum for 30 min on ice. Control cells were stained with the isotype control antibodies. Cells were then resuspended in PBS and analyzed by Accuri C6 (BD Biosciences, USA).

Western blots

Living cells were harvested with lysis solution (50 mM Tris-HCl, 70 mM 2-mercaptoethanol, 2% SDS) supplemented with a cocktail of protease inhibitors and PMSF (Roche Applied Science, USA). Proteins were separated on polyacrylamide gels and transferred by electro blotting onto PDVF membranes, which were further blocked and incubated with primary and secondary antibodies. Proteins were detected with Advance Western Blotting Detection Kit (ThermoFisher Scientific, USA). The antibodies used were listed below: mouse anti-CDH2 (BD Biosciences, USA); mouse anti-CDH1 (BD Biosciences, USA); goat anti-SOX17 (R&D Systems, USA); mouse anti-SNAI1 (Cell Signaling Technology, USA); HRP-conjugated GAPDH (KANGCHENG, China); HRP-conjugated goat anti-rabbit (KANGCHENG, China); HRP-conjugated goat anti-mouse (KANGCHENG, China).

Statistical analysis

Data were analyzed using SPSS11.0 software package and were presented as mean \pm standard deviation (s.d.). Unpaired two-tailed Student's *t*-test was carried out and $P < 0.05$ was considered statistically significant.

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