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FGF signaling via MAPK is required early and improves Activin A-induced definitive endoderm formation from human embryonic stem cells

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ABSTRACT

Considering their unlimited proliferation and pluripotency properties, human embryonic stem cells (hESCs) constitute a promising resource applicable for cell replacement therapy. To facilitate this clinical translation, it is critical to study and understand the early stage of hESCs differentiation wherein germ layers are defined. In this study, we examined the role of FGF signaling in Activin A-induced definitive endoderm (DE) differentiation in the absence of supplemented animal serum. We found that activated FGF/MAPK signaling is required at the early time point of Activin A-induced DE formation. In addition, FGF activation increased the number of DE cells compared to Activin A alone. These DE cells could further differentiate into PDX1 and NKX6.1 positive pancreatic progenitors in vitro. We conclude that Activin A combined with FGF/MAPK signaling efficiently induce DE cells in the absence of serum. These findings improve our understanding of human endoderm formation, and constitute a step forward in the generation of clinical grade hESCs progenies for cell therapy.

1. Introduction

Human embryonic stem cells (hESCs) have a potential application in cell replacement therapy of chronic diseases like diabetes. They can also serve as a study model for early human development or to derive human differentiated cells for research or drug testing. For tissues of endodermal origin, the inability to efficiently induce definitive endoderm (DE) formation in vitro withholds clinical implementation of hESC-based therapy or other applications.

DE is an epiblast-derived primary germ layer, which generates the major internal organs including liver, pancreas, intestines, lungs, and thyroid [1]. Efficient differentiation of hESC into DE was achieved with high concentrations of Activin A [2] combined first with Wnt3a and then with fetal calf serum [3]. This protocol converts about 80% cultured hESCs into DE cells via a mesendoderm intermediate. Although this represents the current standard DE induction protocol, the contribution of unknown factors in serum remains to be addressed so as to precisely understand the developmental regulation of DE, but also to exclude animal products in consideration of clinical application. To this end, and with the objective of optimizing protocols for producing desired cell types from hESCs, assessment of signaling pathways involved in DE differentiation is very useful.

Recent studies showed that transforming growth factor beta (TGF-beta), Wnt and fibroblast growth factor (FGF) are involved in gastrulation and embryonic patterning, and that they also regulate embryonic stem cell differentiation in vitro [4]. The TGF-beta superfamily member Activin A shares the same receptor with Nodal and signals via Smad2/3. It is used in vitro for germ layer determination, with high concentration inducing endoderm while low concentration favors mesoderm differentiation [2,5]. FGF activates specific tyrosine kinase receptors, followed by downstream stimulation of MAPK and PI3K pathways. Basic FGF (FGF-2) was first identified as a mesoderm inducer [6]. Although studies in frog [7,8], zebrafish [9] and mouse [10] demonstrated that mesoderm formation required cooperation between FGF and Activin/Nodal signaling, less attention was paid to the role of FGF signaling in higher vertebrates endoderm formation [11].

The role of Activin A/Nodal, BMP and Wnt signaling in germ layers induction is conserved in lower and higher vertebrates [2,12,13]. Activin A/Nodal is mainly involved in anterior primitive streak (PS) induction whereas BMP and Wnt attenuate endodermal but improve posterior PS induction. On the contrary, FGF contribution remains controversial among species and studies. For instance, recent reports indicate that interdependent FGF/Activin A or FGF/

Abbreviations: AA, Activin A; Cyclo, KAAD-cyclopamine; DE, definitive endoderm; F, FGF2; HBPI, hepatocyte blockade and pancreas induction stage; hESCs, human embryonic stem cells; LY, LY294002; NG, Noggin; PNP, PDX1- and NKX6.1-positive pancreatic progenitor; PPP, PDX1-positive pancreatic endoderm; PS, primitive streak; RA, retinoic acid; SU, SU5402; U, U0126.

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Activin A/BMP signaling promotes endoderm differentiation in mouse and human embryonic stem cells [13–16]. These data are thus at stake with improved endoderm differentiation in lower vertebrates upon FGF inhibition [12]. Additionally, whereas PI3K inhibition improves activin A-inducded DE formation in hESCs [17], it blocks anterior PS induction in the context of Wnt overstimulation [18]. These observations suggest that beside the basic knowledge that graded TGF-beta signaling segregates mesoderm and endoderm from the mesendoderm, there is accumulating evidence that alternative pathways (for instance FGF) control its extent. The exact requirement, nature and timing of FGF signaling has not been addressed during DE induction, despite the knowledge that FGF regulates the patterning of hESCs-derived DE [19]. This knowledge gap promoted us to investigate the potential role of FGF in DE formation from hESCs. We found that FGF signaling via the MAPK/ERK1/2 pathway is required in early stages of DE differentiation. Combining Activin A with transient FGF-2 treatment without serum efficiently generated DE cells, which were further directed towards pancreatic progenitors.

2. Materials and methods

2.1. Maintenance and differentiation of hESCs

The VUB07 and VUB14 lines were generated and characterized at our institute [20], and registered at the European Human Embryonic Stem Cell Registry. The HSF6 line was initially derived by the University of San Francisco, California. Cells were maintained undifferentiated and manually passaged as previously described [20]. The data reported here are mainly from the VUB07 cell line, while the VUB14 and HSF6 lines were only used to study the temporal FGF effects.

For DE differentiation, hESCs were seeded onto feeders as usual or cultured on Matrigel (1/20 dilution; BD Biosciences, Franklin Lakes, NJ) in feeders-conditioned medium. Cultures at 80% confluence were thoroughly washed and induced in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml Penicillin, 100 μg/ml Streptomycin and 100 ng/ml Activin A (R&D Systems, Minneapolis, MN) for 3 days (VUB07, VUB14) or 4 days (HSF6). The 3-day culture did not efficiently induce DE in HSF6 line; however, a 4-day treatment led to the characteristic DE morphology and significant upregulation of DE markers (Supplementary Fig. 2). Wnt3a (25 ng/ml; R&D Systems), FBS (0.2%; PAA Laboratories, Pasching, Austria), FGF-2 (50 ng/ml; Invitrogen), SU5402 (5 μM; Calbiochem/Merck, Darmstadt, Germany), U0126 (5 μM; Sigma, Saint Louis, MO), or LY294002 (2 μM; Calbiochem/Merck) was added at indicated periods.

For further differentiation towards pancreatic fate, the previously described methods [20] were used with slight modifications as shown in Supplementary Table 1.

2.2. Western blot

Total protein fraction was extracted in radioimmunoprecipitation buffer (RIPA) supplemented with a protease inhibitors complex and the concentrations determined by the BCA method on the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Protein extracts (25 μg) were separated on 7–10% SDS–polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) before blotting onto nitrocellulose membranes. Membranes were blocked with Sea Block blocking buffer (Thermo Fisher Scientific, Rockford, IL) and exposed to primary antibodies overnight at 4 °C. Membranes were washed and further exposed for 1 h to HRP-conjugated anti-goat, anti-rabbit or anti-mouse (1/1000 dilution) secondary antibodies at room temperature. Detection was performed by chemiluminescence on Kodak films. Primary antibodies used for Western blot were goat-anti beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-AKT, rabbit anti-pAKT, rabbit anti-MAPK and mouse anti-pMAPK (all used at 1/1000 dilution, all from Cell Signaling Technology, Danvers, MA).

2.3. Quantitative real-time PCR

Total RNA extraction, reverse-transcription and real-time PCR were performed as we described before [21]. The sequences of the primers used are listed in Supplementary Table 2. Relative quantification of gene expression was calculated by the ddCt method and normalized to endogenous control (*TBP*). Data are presented as fold change versus undifferentiated hESCs or control condition (Activin A alone).

2.4. Immunofluorescence

Cultured cells were processed for immunofluorescence as previously described [21]. The following primary antibodies were used: goat anti-GSC, 1/50 (Santa Cruz Biotechnology; SC-22234), goat anti-FOXA2, 1/200 (Santa Cruz Biotechnology; SC-9187); mouse anti-SOX17, 1/100 (R&D Systems; MAB1924); goat anti-PDX1, 1/100 (R&D Systems; AF2419); mouse anti-NKX6.1, 1/250 (Developmental Studies Hybridoma Bank, Iowa; F55A10). Secondary antibodies were Alexa Fluor 488- and 555-conjugated donkey anti-goat or mouse, 1/500 (Invitrogen); TRITC-labeled donkey anti-mouse, 1/100 (Jackson ImmunoResearch Laboratories, West Grove, PA). The proportions of GSC, SOX17 or FOXA2 positive cells were determined by manual cell counting from five randomly selected areas under the fluorescence microscope.

2.5. Statistical analysis

Data from at least three independent experiments were analyzed by one-way ANOVA with Bonferroni's multiple comparison post hoc test (Prism 5, GraphPad Software Inc., La Jolla, CA) and expressed as mean ± SEM. Differences observed were considered statistically significant at the 5% level and were displayed on the figures as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. FGF signaling is involved in Activin A-induced DE differentiation

Consistent with the knowledge that Activin A/Nodal signaling induce DE in mammals, hESCs treated with high concentration of Activin A in serum-free medium differentiated into DE as evidenced by the increased *SOX17* and *FOXA2* expression. The mesendoderm and anterior PS markers *BRY* and *GSC* were transiently and sequentially increased during the first 2 days of induction (Supplementary Fig. 1A), after which they were reduced with the emergence of DE cells. These data suggest that high concentration of Activin A supplemented for 3 days without serum successfully induced DE markers expression in hESCs. However, their expression levels were roughly 50% lower than achieved with the standard protocol (DMR; see later).

With regards to the data from lower vertebrates [12], we would expect inhibition of DE differentiation by activated FGF signaling and the opposite upon FGF antagonism. To test this hypothesis, FGF signaling was modulated in Activin A-treated hESCs. We first assessed the effects of short time exposure to Activin A with or without FGF receptor agonist (FGF-2) or antagonist (SU5402) on MAPK phosphorylation (Fig. 1A). MAPK phosphorylation was observed in hESCs starved of growth factors for 2 h (Fig. 1A), indicat-

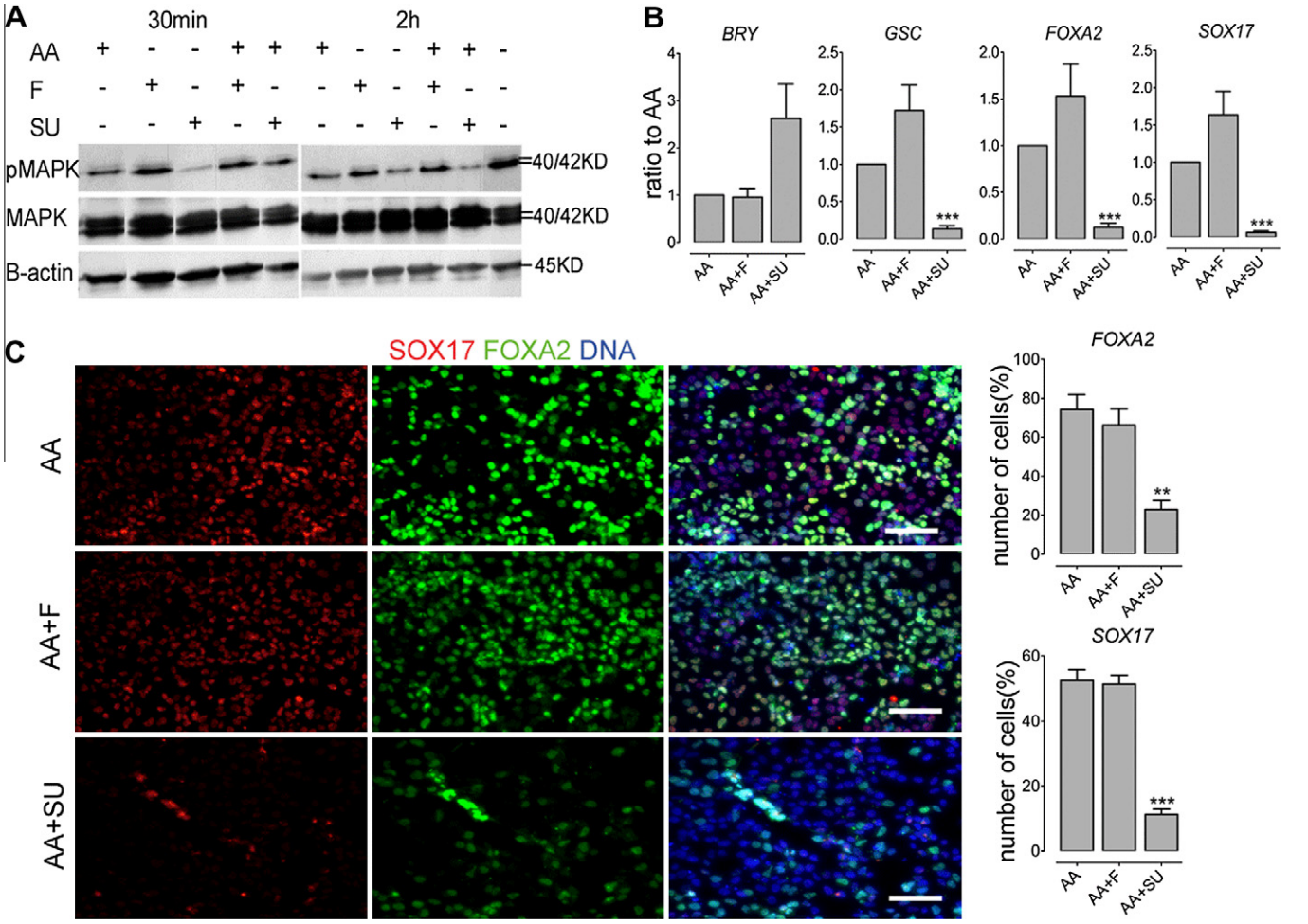

Fig. 1. Inhibition of FGF signaling blocked Activin A-induced DE formation. (A) Assessment of MAPK phosphorylation by Western blot in hESCs treated with or without indicated factors for 30 min and 2 h. (B) Gene expression profile of DE (*GSC, FOXA2, SOX17*) and mesoderm (*BRY*) markers after 3 days exposure to Activin A alone (AA), or with FGF2 (AA + F) or SU5402 (AA + SU). (C) Protein expression profile of DE markers, and the quantification of FOXA2- and SOX17 positive cells after 3 days culture in these conditions. Scale bar: 50 μm.

ing the persistence of the effect of FGF-2 used in hESCs culture medium or its paracrine secretion by feeder cells. This residual MAPK activity was abrogated after SU5402 supplementation for 30 or 120 min. Activin A partially restored MAPK phosphorylation in the presence of SU5402 at 30, but not at 120 min, suggesting an intrinsic stimulatory effect on the FGF pathway. This is consistent with the findings that Activin A maintains hESCs pluripotency by inducing FGF-2 expression [22]. That Activin A failed to restore MAPK phosphorylation at 2 h in the presence of SU5402 might indicate the unresponsiveness of the above mentioned system once differentiation is initiated. Furthermore, FGF-2 addition enhanced MAPK phosphorylation already induced by Activin A. Taken together, these data indicate that FGF signaling can be modulated in the course of Activin A-induced DE differentiation.

Next, we examined the influence of FGF modulation on DE differentiation *per se*. Cells were exposed to Activin A alone and either with FGF-2 or SU5402 for 3 days without serum, then RT-PCR was performed for *BRY, GSC*, *SOX17* and *FOXA2*. Addition of SU5402 to Activin A severely reduced DE markers expression on day 3. On the contrary, this treatment led to an increased expression of the mesendoderm/mesoderm marker *BRY* (Fig. 1B). In agreement with the effect of SU5402, combination of FGF-2 and Activin A slightly, but reproducibly increased (50 to 70%) DE markers without affecting *BRY* levels (Fig. 1B). In addition, neither activation nor inhibition of FGF signaling influenced the generation of alternative fates such as ectoderm (*SOX1*) and visceral endoderm (*SOX7*) (Supplementary Fig. 1B). Immunofluorescence analysis confirmed these findings at the protein level, detecting GSC- (61.09 ± 8.16% in AA; 68.03 ± 6.68% in AF), FOXA2- (74.27 ± 7.69% in AA; 66.32 ± 8.33% in AF) and SOX17- (52.48 ± 3.30% in AA; 51.30 ± 2.78% in AF) positive cells except in SU5402-treated condition (5.81 ± 1.71% GSC+; 22.90 ± 4.58% FOXA2+; 11.27 ± 1.64% SOX17+) (Fig. 1C). All together, our data indicate that FGF inhibition severely attenuated DE induction from Activin A-treated hESCs, and that its stimulation slightly increased DE, but not mesoderm markers expression.

3.2. Derivation of DE from hESCs has an early dependence on FGF signaling

Embryo development events involve not only specific molecules acting in defined compartments, but also their correct timing. We aimed at defining the time window of the observed FGF requirement during DE formation. For this purpose, cells were exposed to Activin A for 3 days, combined with FGF-2 or SU5402 for the first day (0–1), for the last day (2–3) or throughout the 3 days (0–3). Expression of DE markers *GSC*, *FOXA2*, *SOX17* and *CXCR4* increased about twofold versus Activin A alone after FGF-2 addition during the first day only, but not in the other conditions. On the contrary,

BRY expression furhter decreased only when cells received FGF-2 at early time points (Fig. 2A). These findings indicate that transient and early supplementation of FGF-2 to Activin A specifically improves DE markers expression from hESCs.

Accordingly, SU5402 supplementation on the first day or during the 3 days of Activin A treatment significantly decreased DE markers expression and slightly increased *BRY* levels. Furthermore, these genes were not significantly affected by SU5402 applied only on the last day of differentiation (Fig. 2A). These latter findings support the hypothesis that active FGF signaling is no longer essential in the final stage of DE induction. Therefore, our data indicate a positive FGF signaling effect on the initial formation of DE on one hand, and suggest a function in the establishment of mesendoderm progenitors and their differentiation towards DE cells.

Several aspects of FGF modulation illustrated in VUB07 cell line could be reproduced in HSF6 cell line (Supplementary Fig. 3A). On the contrary, addition of exogenous FGF-2 did not increase DE gene expression in the VUB14 line and 3 days inhibition of the pathway just marginally reduced DE markers (Supplementary Fig. 3B). To unravel this discrepancy, we evaluated the basal and stimulated levels of FGF downstream effectors phospho-MAPK and phospho-PI3K/AKT in these cells. We observed a higher MAPK-, but not PI3K phosphorylation, in the VUB14 versus VUB07 either in the presence of FGF-2 or following FGF-2 removal for 24 h (Supplementary Fig. 3C). This supports a higher level of endogenous (autocrine/paracrine) FGF signaling in VUB14 and explains at least in part, its lack of response to FGF supplementation. Alternatively, VUB14 cells could display much more FGF receptors on their membrane, so that the SU5402 concentration used was not enough to block all of them. Unfortunately, we could not test this hypothesis as higher SU5402 concentrations compromised cell viability.

3.3. The MAPK/ERK1/2 rather than the PI3K/AKT pathway is required for DE induction

We aimed at deciphering which of the two major intracellular pathways downstream of FGF signaling (ERK1/2 mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3 kinase (PI3K)) was involved in the observed FGF requirement early during DE formation. We used two small molecules, U0126 (MAPK inhibitor) and LY294002 (PI3K inhibitor) for this purpose. In the presence of U0126, all tested DE markers were reduced to similar levels as with SU5402 treatment (Fig. 2B), and the visceral endoderm marker *SOX7* was slightly increased (Supplementary Fig. 1C). LY294002 did not reproduce all these findings, but only *FOXA2* expression was moderately reduced. U0126 dramatically reduced the number of SOX17 and FOXA2 positive cells versus Activin A alone or with LY294002 (Fig. 2C). Considering the abrogation of MAPK phosphorylation in Activin A + SU5402 condition (Fig. 1A), and the similar outcomes of Activin A + SU5402 and Activin A + U0126 treatments (Fig. 2), we conclude that active MAPK signaling is required for DE induction by Activin A.

3.4. Differentiation of pancreatic progenitors from DE cells induced in serum-free medium by Activin A and FGF-2

The current standard protocol (DMR) for inducing DE includes low amounts of fetal calf serum in the last two days. It generates at least 60% DE cells co-expressing SOX17 and FOXA2, and is widely used for deriving endoderm progenies from hESCs [3,20]. However, the supplemented animal serum constitutes a limitation for clinical implementation of generated progenies, but also for experimental reproducibility given the undefined composition and unpredictable batch-to-batch variations of the serum. We obtained efficient DE formation comparable to DMR protocol in ser-

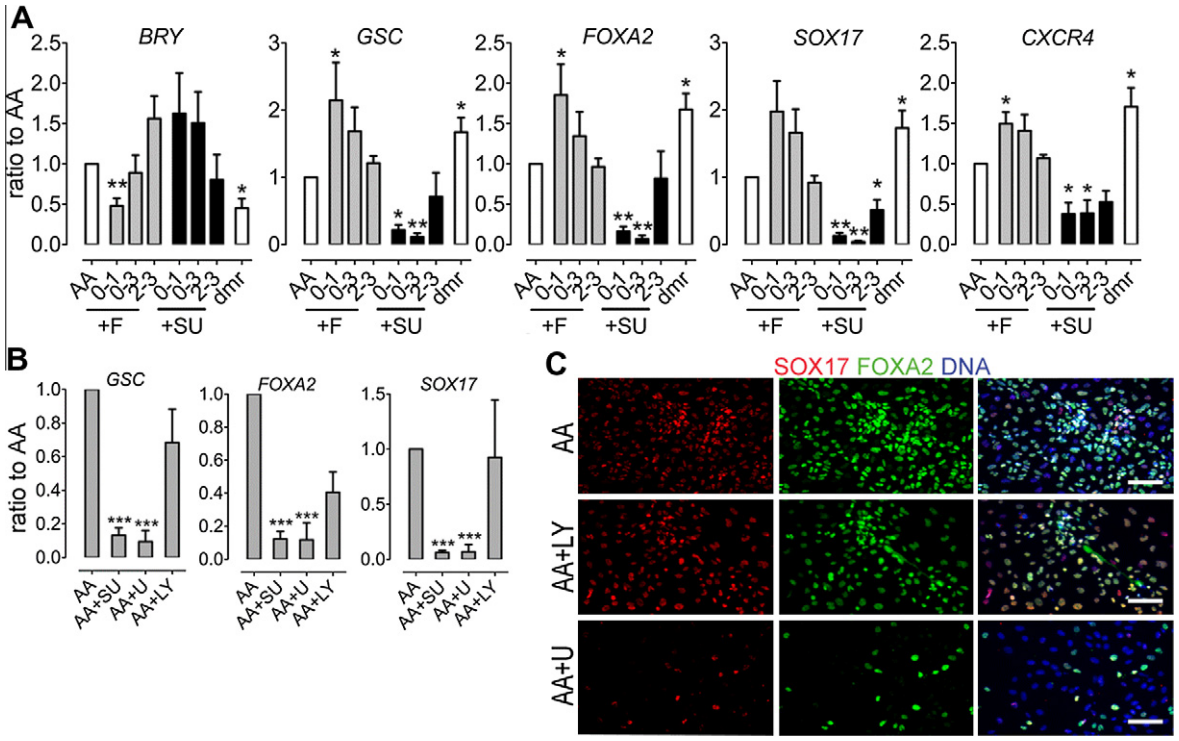

Fig. 2. FGF signaling via MAPK is required during early stages of Activin A-induced DE formation. (A) Expression of *GSC*, *FOXA2*, *SOX17*, *CxCR4* and *BRY* on day 3 in the VUB07 cell line after AA treatment and temporal modulation of FGF signaling with F and SU. A bar corresponding to the standard protocol (dmr) is added for comparison. (B) RT-PCR analysis of DE markers expression after AA treatment with or without FGF inhibitors revealed the involvement of MAPK/ERK1/2, but not PI3K pathway. (C) Immunofluorescence analysis of SOX17 and FOXA2 after AA treatment with or without FGF inhibitors. U, U0126; LY, LY294002. Scale bar: 50 μm.

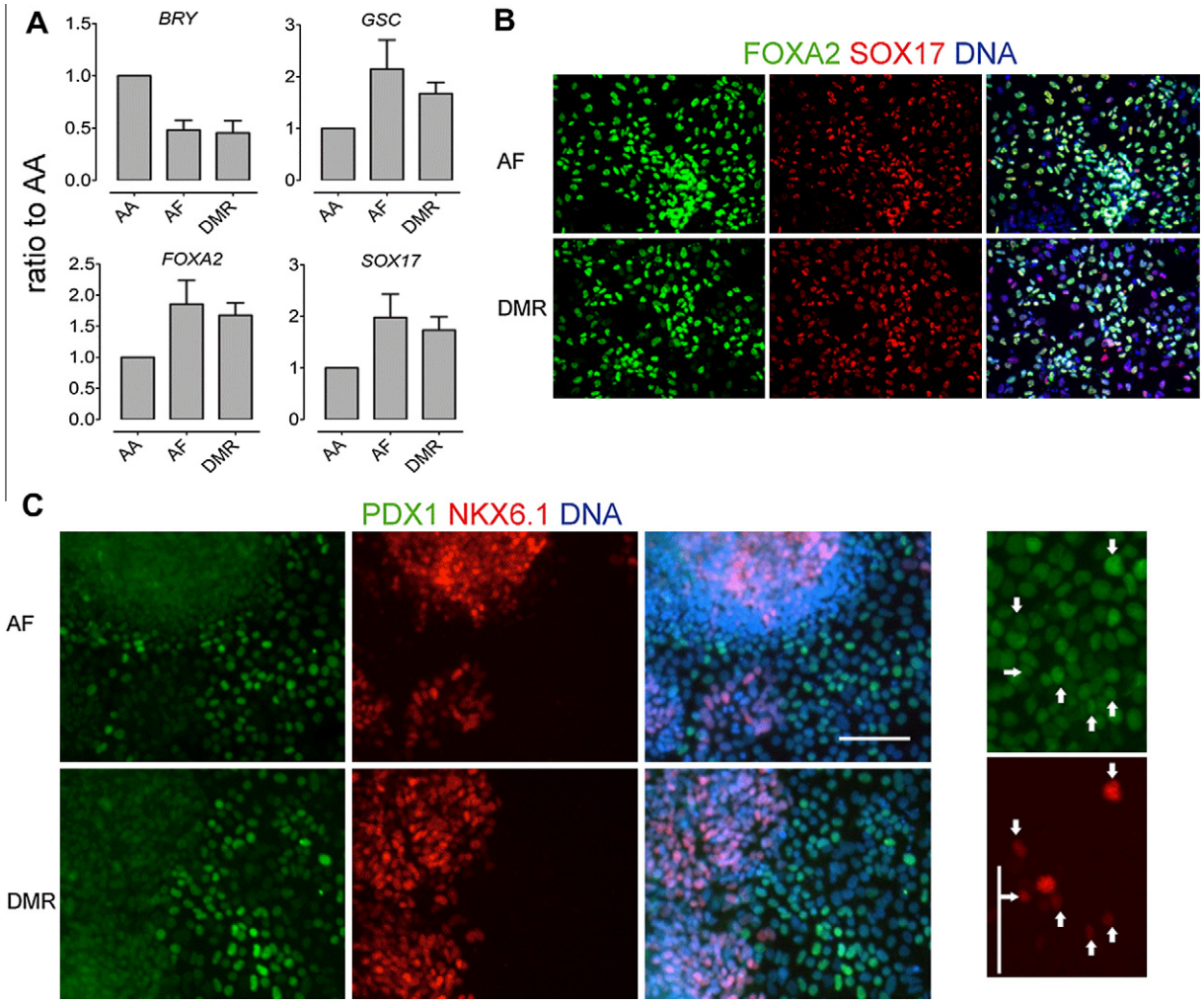

Fig. 3. In vitro derivation of pancreatic progenitors from DE cells induced by DMR or AF protocols. (A) Similar profiles of *GSC*, *FOXA2*, *SOX17* and *BRY* gene expression were found between AF and DMR protocols at day 3. (B) Coexpression of SOX17 and FOXA2 was confirmed in about 60% of differentiated cells. (C) Both DMR- and AF-derived DE differentiated into PDX1^{high}NKX6.1^{negative}, PDX1^{low}NKX6.1^{high} and PDX1^{high}NKX6.1^{high} cells (marked by arrows) in vitro. Scale bar: 50 m.

um-free medium by combining Activin A (day 0–3) with temporal stimulation (day 0–1) of FGF signaling (AF). Both methods showed similar levels of DE markers induction at transcripts and at proteins levels (Fig. 3A and B). Therefore, FGF-2 early treatment during Activin A-induced DE formation is also efficient in the absence of serum supplementation.

We finally examined the differentiation potential of AF-induced DE cells by subjecting them to our recently developed methods for deriving pancreatic endoderm from hESCs [20] with some modifications. Indeed, pancreatic progenitor cells that are characterized by expression of PDX1 and NKX6.1 could be obtained at the PNP stage with both DE induction protocols, and these cells were PDX1^{high}NKX6.1^{negative} (±40%), PDX1^{low}NKX6.1^{high} (±40%) and few were PDX1^{high}NKX6.1^{high} (<1%) (Fig. 3C).

4. Discussion

Several studies implicated FGF signaling in mesoderm formation, but only few critically assessed its function in the control of endoderm formation. With regards to hESCs, much attention was focussed on generating "terminally" differentiated cells or tissues. In this work, we used the hESCs model and analyzed how FGF pathway interacts with Activin A signaling during DE formation. We found that blocking FGF signaling with SU5402 abolished Activin A-induced DE formation, which is at stake with the findings in zebrafish where active FGF signaling represses Nodal-induced endoderm differentiation [12]. Activin A signaling involves SMAD2 phosphorylation. As SMAD2 phosphorylation is not affected in hESCs treated with Activin A and SU5402 [23], the attenuation of DE differentiation upon FGF blockade is unlikely a consequence of limited SMAD2 activity. Furthermore, although SU5402 blocked Activin A-induced DE differentiation, Activin A did not significantly activate the downstream FGF effector MAPK. Therefore, activated FGF signaling is not a primary pathway, but a concomittant requirement for DE induction from hESCs in response to Activin A. We observed that combining Activin A with FGF-2 increased DE markers expression by 50% versus Activin A alone, suggesting that FGF-2 potentiates DE induction by Activin A, possibly via ERK2-dependent phosphorylation of SMAD2 as previously suggested [24] or/and via activation of Wnt signaling, which improves mesendoderm induction and is thought to be regulated by MAPK [3]. Considering that Activin A induced DE in a FGF-dependent manner, and that FGF failed to induce DE in the absence of Activin A (data not shown), there must exist a synergy between these pathways in our hESCs model.

A recent study in mESCs suggested a late dependence of DE induction on FGF signaling [14]. In the present study with Activin

A-induced hESCs, we rather found an early dependence on FGF signaling for DE markers expression. We also noticed a slight but significant decrease in *SOX17* expression following late FGF inhibition. These discrepancies might be related to the known species differences in the integration of molecular signals. To clarify this issue, it would be worth investigating mouse epiblast stem cells, which share similar properties with hESCs [13]. While awaiting such data, it appears obvious for now that a short period of active FGF signaling is necessary and required in hESCs to shift from an undifferentiated status to an endoderm fate in the presence of Activin A. This observation parallels other findings that FGF/MAPK signaling is required, but transiently, for switching on mESCs differentiation [25,26] and for setting up neural progenitors during the first 17 to 24 h of induction [26].

By making use of the small molecules U0126 and LY294002, we showed that the MAPK pathway plays a dominant role in DE induction. Previous studies indicated that PI3K antagonizes the ability of hESCs to differentiate into DE in response to TGF-beta signals [17]. We did not observe any improvement of DE differentiation in the presence of the PI3K inhibitor LY294002. It is possible that our serum-free condition, together with the brief washing step that preceded DE induction eliminated the negative effect of PI3K signaling normally activated by insulin from the hESC maintenance medium.

In *Xenopus*, the mesoderm-inducing capacity of Activin A requires endogenous FGF signaling which contributes by regulating T box transcript level [7,8]. In the model presented here, expression of the mesoderm gene *BRY* was slightly increased upon FGF inhibition. Although previous studies showed that FGF-2 could antagonize BMP4 activity in hESCs and that *BRY* is a target of BMP signaling [14], the slight increase we observed following SU5402 treatment could not be accounted for BMP since it was not affected by addition of the BMP inhibitor Noggin (data not shown).

In conclusion, our studies have clarified the role of FGF signaling during Activin A-induced DE formation in hESCs. Combining Activin A and FGF-2 during the first day of differentiation efficiently generated DE cells in serum-free condition, reaching the same level as the current standard protocol that includes serum supplementation. Furthermore, DE cells generated by these means were directed towards a pancreatic endoderm fate in vitro, which showed potential to further generate NGN3-positive endocrine progenitors and Insulin/C-peptide-positive beta cells after 12 weeks transplantation in vivo ([21] and manuscript in preparation). These observations are valuable for our understanding of early human embryo development and provide a prospective way for developing safe materials in view of future cell replacement applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.098.

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