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ORIGINAL ARTICLE

Induction of primitive streak and mesendoderm formation in monolayer hESC culture by activation of TGF- β signaling pathway by Activin B



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Abstract Human embryonic stem cells (hESCs) have the ability to differentiate into all human cells, however controlling the differentiation has always been a challenge. In the present study we have investigated the direct differentiation of hESCs on MEFs by using TGF- β signaling pathway activators Activin A and Activin B. Activation of the TGF- β pathway with Activin B in low serum highly induced primitive streak and mesendoderm formation after 24 h, which included up-regulation of SOX 17 and BRACHYURY protein and gene expression. Continuous stimulation with Activin B in 2% serum further induced mesendoderm formation by increased gene expression of Brachyury, SOX17, MEOX and FOX at the same time we found down-regulation of neuroectodermal marker genes. Further, by stimulating the mesodermal cells by BMP-2 we succeeded to induce mesenchymal like cells with high expression of mesenchymal markers including; MEOX, FOX, RUNX2, COL1 and OSTEOPONTIN. In conclusion we have directed the differentiation of hESCs as monolayer to primitive streak like cells with Activin B and further into pure mesoderm and mesenchymal like cells by BMP-2.

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1. Introduction

Mesoderm is the middle germ layer during embryonic development, and vertebrate mesoderm produces a wide range of tissues including, the muscle, and the circulatory system including the heart, vasculature, hematopoietic system, kidney, gonads, dermis, cartilage and the bones. In lower vertebrates, it is well known that definitive endoderm and mesoderm cells arise from a common bi-potent mesendoderm progenitor. Recently, it has been suggested that such a mesendoderm

progenitor also exists in mammalian embryos (Lickert et al., 2002; Rodaway and Patient, 2001; Tada et al., 2005). There are some essential signaling pathways in gastrulation and primitive streak formation that are activated by growth factors such as Nodal, BMP/TGF- β and WNT for initiation of gastrulation (Park, 2011; Sowa et al., 2002). Ligands for the signaling pathways are also expressed in the primitive streak (PS) and node, which is essential for the accurate mesoderm and definitive endoderm development (Wolfe and Downs, 2014).

Monolayer human ESC differentiation is the simplest approach for direct differentiation. hESCs grown either on feeders or feeder free layers (using a matrix) can be induced to differentiate into specific lineages (Bauwens et al., 2008; Cameron et al., 2006; Keller, 2005; van den Berg et al., 2015). Differentiation on known substrates can minimize the influence of neighboring cells so that the true effect of growth factors/cytokines can be measured (Yao et al., 2006). A study from the group of Kevin D'Amour has shown that stimulation of TGF- β signaling by Activin induction promotes hESC differentiation into definitive endoderm through a transition state of primitive streak when grown as a monolayer (D'Amour et al., 2005). In their study they demonstrated that removal of Activin and introduction of serum after 3 days of stimulation gave rise to mesoderm and inhibited endoderm differentiation. To examine their procedure for mesodermal differentiation we aimed to differentiate hESCs as a monolayer in the presence of Activin for induction of primitive streak formation.

2. Materials and methods

2.1. Culturing of undifferentiated hESC cells

All tissue culturing was carried out using the sterile technique under laminar flow conditions. Cells were grown at 37 °C in an atmosphere of 5% CO₂ and a relative humidity of 90–100%. The undifferentiated hESC cell line used during this study was HUES9, which was obtained from Howard Hughes Medical Institute, Harvard University. Undifferentiated hESC cells were maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblast (MEF) cells in Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) (Invitrogen) supplemented with 15% Knockout Serum Replacement (KO-SR) (Invitrogen), 0.5% human serum albumin (ZLB Behring), 0.1 mM β -mercaptoethanol (Sigma), 1% MEM non-essential amino acids solution (Invitrogen), 2 mM glutamine as Glutamax-I (Invitrogen), 50 units/ml penicillin and 50 μ g/ml streptomycin (Invitrogen), and 5–10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen). Before plating of cells, tissue culture plates (Nunc) were coated with 0.1% gelatine (Sigma) for approximately 30 min at room temperature (RT). The MEF cells were expanded in KO-DMEM supplemented with 10% fetal bovine serum (FBS) (PAA), 2 mM Glutamax-I, 50 units/ml penicillin, and 50 μ g/ml streptomycin. MEF cells were mitotically inactivated by γ -irradiation. Prior to inactivation, the MEF cells were washed once with D-PBS^{-/-} (Invitrogen) chelating Mg²⁺ and Ca²⁺ from adhesive proteins followed by enzymatic dissociation with 0.05% trypsin with 0.53 mM EDTA (Invitrogen) for 1–2 min at 37 °C. Trypsin

was removed and inactivation was carried out by γ -irradiation for 15 min at 266.7 rads/min. The MEF cells were stored in vials of 1.5×10^6 and 5×10^6 cells in 0.5 ml freeze media (90% MEF media and 10% dimethyl sulfoxide (DMSO) (Sigma)) at -150 °C.

One day in advance of passaging the hESC cells, plates with MEFs were prepared by seeding 20,000 cells/cm² MEF cells onto gelatin pre-coated plates. The MEF cells were allowed to attach for approximately 24 h, after which media was changed to hESC media for at least 3–4 h or overnight (O.N.) for conditioning hESC media. Human ESC colonies were passaged at 1:3–1:6 split ratio every 4–7 days by trypsinization. When cultures reached sub-confluence, the colonies were gently washed with PBS^{-/-} and 0.05% trypsin/EDTA was added. The detachment was observed under the microscope. When the MEF cells had rounded up and mostly detached, trypsin was aspirated and pre-warmed hESC media was added to the colonies. Human ESC colonies were scraped off the dishes and gently pipetted up and down to break up large clumps containing 50–100 cells. Subsequent cell suspension was transferred to the pre-prepared MEF culture plates containing conditioned hESC media. In between split days, a half media change was performed every day.

2.2. Monolayer differentiation of hESCs to mesenchymal cells

Human ESCs were differentiated into definitive mesendoderm cells as monolayer. The differentiation protocol was derived from D'Amour et al. (2005). Differentiation was carried out in RPMI 1640 media (Invitrogen), supplanted with 2 mM Glutamax-1, 1% penicillin/streptomycin, and FBS at concentrations ranging from 0.5% to 2.0%. To induce mesendoderm differentiation 100 ng/ml of human Activin A or B was added to differentiation media. When hESC cultures reached approximately a confluency of 80–90%, differentiation was initiated. Before replacing media with differentiation media, the cells were gently washed twice with PBS^{+/+} to remove any growth factors bound to cell surface receptors. For the first 24 h, differentiation media with 0.5% FBS were used. The subsequent 24–96 h of differentiation cells were cultured with 2.0% FBS. To promote mesenchymal formation, 72 h of pre-differentiated mesendoderm cells were subjected to 100 ng/ml of human recombinant BMP2 (R&D) for additional 6–9 days.

2.3. RNA extraction, reverse transcription and real-time PCR

Total RNA from cell samples was isolated using the TRIZOL method according to the manufacturer's instructions. RNA quantitation was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop). cDNA was synthesized from 1 to 2 mg of total RNA using a commercial revertAid H minus first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Real-time qPCR was performed on a MyiCycler thermal cycler (Bio-Rad, Copenhagen, Denmark) using iQ SYBR Green mastermix (Bio-Rad) according to the manufacturer's instructions. Quantification of target and reference genes (β -actin) was performed in duplicate. Following normalization to the β -actin gene, expression levels for each target gene were calculated using the comparative threshold cycle (CT) method (1/2^{DCT}, where DCT is the

difference between CT target and CT reference). Data were analyzed using the Optical System Software Version 3.1 (Bio-Rad) and Microsoft Excel 2000 (Seattle, WA, USA) to generate relative expression values. Primers used in this study are listed below with employing an annealing temperature of 60 °C. Data are presented as the mean \pm SD from three independent experiments. Primers used were OCT4: forward GAAGGTATTTCAGCCAAAC, reverse CTTAATCCCAA AACCTG, SOX17: forward CAGCAGAATCCAGACCT GCA, reverse GTCAGCGCCTTCCACGACT, MEOX1: forward AATCATCCAGGCGGAGAAA, reverse AAGGCCG TCCTCTCCTTG, FOXF-1: forward CAGCCTCTCCACG CACTC, reverse CCTTTCGGTCACACATGCT, NEUROD1: forward CTTGGCCAAGAACTACATCTGG, reverse GGA GTAGGGATGCACCGGAA, BRACHYURY: forward GAAGGTGGATCTCAGGTA GCG and reverse CGCAG CTGGGCGCCTGCGGCT.

2.4. Immunocytochemical staining

For immunostaining, cells were grown to 80–90% confluence on 2-, 4- and 8-well Permanox chamber slides (Nunc, Roskilde, Denmark). The slides were fixed in 4% formaldehyde for 10 min and then washed three times in PBS–/–. Nonspecific immunoglobulin G binding sites were blocked for 20 min with 5% bovine serum albumin (BSA) in PBS or serum-free block solution (Zymed, San Francisco, CA). Primary, as well as secondary antibodies, were diluted in 2% BSA dissolved in PBS. Cells were incubated with primary antibodies for 60 min, antibodies used were goat anti-human Brachyury (R&D, 1:100) and goat anti-Human Sox 17 (R&D, 1:400). Unbound antibody was removed by 3 \times 2 min of washing with TBS, before biotinylated secondary antibody was added for 10 min allowing it to bind specifically to the Fc domain of the primary antibody. Unbound antibody was removed by rinsing 3 \times 2 min with TBS, followed by incubation with streptavidin–peroxidase conjugate for 10 min, resulting in the formation of biotin/streptavidin complex. Cells were again rinsed 3 \times 2 min with TBS, after which the chromogen, AEC Substrate (Zymed), was added. The AEC (aminoethyl carbazole) chromogen was incubated with the cells for 3–5 min, until adequate color development was seen. The peroxidase color reaction was halted by washing twice with distilled water. Counter staining of the nucleus was carried out by hematoxylin staining for 30 s. Cells were again rinsed twice with distilled water and finally mounted with Aquatex.

2.5. Statistical analysis

All results are presented with the standard deviation (SD) of at least 3 independent experiments. Two-tailed Student's *t*-tests were used for testing differences between groups. *P* values < 0.05 were considered significant.

3. Results and discussion

Using defined conditions Kubo et al. were the first to show that mESC differentiated into definitive endoderm by culturing them in the presence of Activin A under serum free conditions. Furthermore, they were able to show that the endoderm cells developed from a bi-potent BRACHYURY+ mesendoderm

population which, in turn, were able to give rise to both definitive endoderm and mesoderm (Kubo et al., 2004). Furthermore, a recent study has shown that induction of mesoderm progenitor cells by BMP-2 enhances osteogenic differentiation (Ponce et al., 2008), and mESCs respond to BMP2 stimulation and differentiate into osteo-, chondro and adipogenic lineages (Zur Nieden et al., 2005). In order to investigate whether the hESCs are able to differentiate into primitive streak-like cells *in vitro* in response to Activin treatment and further to differentiate into mesoderm in response to BMP-2 treatment, we applied the differentiation protocol as described by D'Amour and colleagues with some modifications (Fig. 1). Confluent undifferentiated hESCs were grown as colonies on inactivated MEF cells, the differentiation was initiated by changing growth media to RPMI based serum free media with continuous exposure to 100 ng/ml Activin B for 5 days (outlined in Fig. 1). We have previously shown that Activin B is a much more potent inducer of primitive streak and endoderm than Activin A in the 3-D embryoid formation differentiation model (Frandsen et al., 2007), therefore Activin B was used throughout the study. During monolayer differentiation we observed that the cell line we used, HUES9, reacted differently to stimulation from Activin A or B. Activin A did not induce BRACHYURY gene expression, whereas Activin B showed high BRACHYURY and SOX17 gene expression (Fig. 2). Therefore, we determined to continue the use of Activin B for further differentiation. For the first 24 h RPMI media was supplemented with 0.5% FBS and the following 4 days of differentiation were carried out in 2.0% FBS. Cells cultured in RPMI media with no Activin B were included as controls.

Three days of induction with Activin B enhances endoderm and mesoderm marker expression. During the first day of treatment cells started to detach, and after 2–3 days, massive cell death was observed. At 3 days of Activin B treatment, cells with a flat, cobblestone-like appearance emerged beneath the

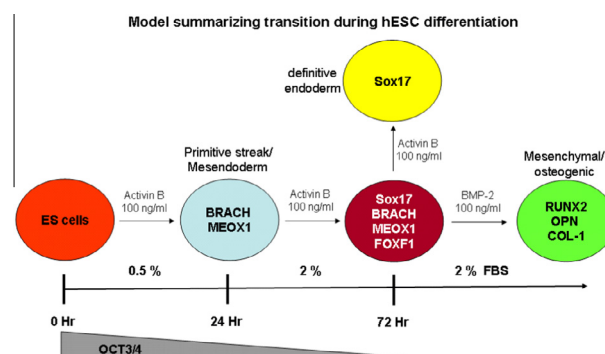


Figure 1 Experimental procedure for directing the differentiation of hESCs toward a mesendodermal population and further into mesoderm. Differentiation of hESCs through a process that closely recapitulates gastrulation can be orchestrated by the application of high dose of Activin B in the context of low serum. Maximal expression of primitive streak-expressed genes (MEOX1, BRACH) occurs at 24–36 h. If maintained in high dose Activin B, the cells are efficiently converted into definitive endoderm cells expressing SOX17. In contrast, if exogenous Activin B is removed and BMP-2 added cells express mesoderm/mesenchymal markers (FOXF1, MEOX1, RUNX2, OP and COL-1).

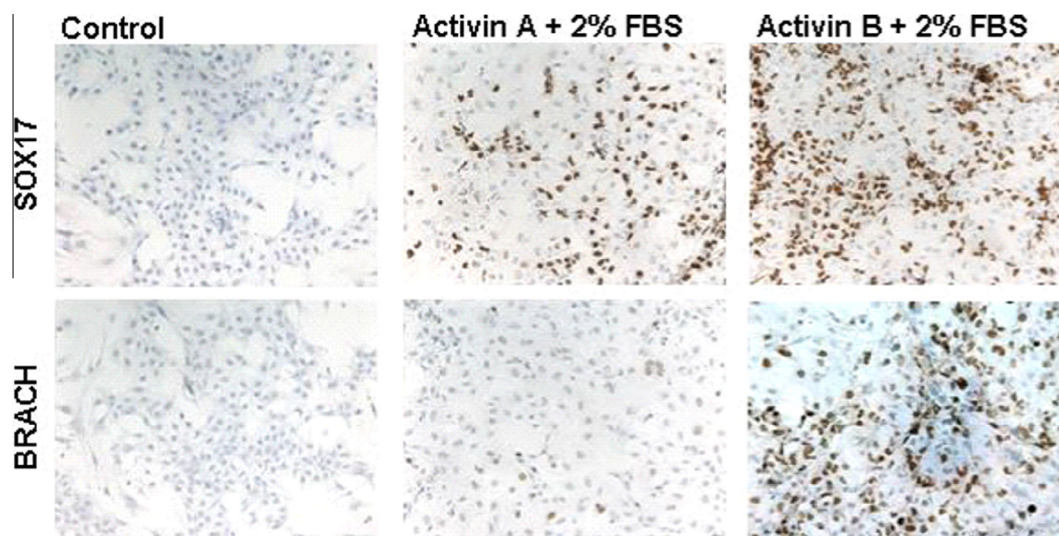


Figure 2 Immunostaining revealing the difference between Activin A and B on mesendoderm differentiation. Human ESCs were differentiated in the presence of 100 ng/ml of either Activin A or B or 72 h in RPMI media containing 0.5% FBS for the initial 24 h and 2% for rest of the differentiation period. A matched control is included without any stimulation. Cells were stained with antibodies specific for SOX17 and brachyury (BRACH), original magnification is 100 \times .

detached cells, forming a flat sheet of cells covering the base of the culture dishes. After 5 days of Activin B treatment, cell cultures were composed of a very homogeneous population with relatively large cobblestone-like cells, which appeared to be very similar to hepatocytes. In the non-treated control cultures, massive detachment of cells was also observed during the five days of induction, however, no cells with cobblestone-like morphology were observed (Fig. 3A).

Activin B stimulation of hESCs in media containing a low percentage of serum initiated differentiation of the cells, 24 h after stimulation the cells started to express the primitive streak and mesoderm marker BRACHYURY and the endoderm marker SOX17, although fewer cells expressed SOX17 than BRACHYURY. The expression of both markers increased gradually, and expression of BRACHYURY reached its maximum at 36 h of Activin B treatment, hereafter the expression level decreased even though continuous Activin B stimulation was continued. Conversely, SOX17 protein expression increased until five days of Activin B induction (Fig. 2A). Gene expression analysis demonstrated that the pluripotency marker Oct4 was rapidly down regulated in serum free media. However, when cells were stimulated with Activin B they did not show the same level of OCT4 down-regulation. Recent papers have indicated that activation of Activin/Nodal signaling is important for the maintenance of the pluripotent state of hESCs (James et al., 2005; Vallier et al., 2005); these data could demonstrate why a late down-regulation of Oct-4 was seen in Activin stimulated monolayer cultures. Furthermore, gene expression analysis showed consistent agreement with immunostaining analysis with a gradual increase in BRACHYURY expression reaching a maximum at day 3, and again, SOX17 expression showed a maximum level of expression after 5-days of Activin B induction (Fig. 2B). The early mesoderm marker MEOX1 (Candia et al., 1992) was shown to be highly induced by Activin B induction for the first 3 days, hereafter the expression level decreased dramatically, concurrently a later mesoderm

marker, FOXF1, was only seen to be expressed at day 3 with the expression level decreasing at day 5 (Fig. 2B). To look for neuroectodermal differentiation, we monitored the expression of the early ectoderm marker NEUROD1, this was only expressed at day 3 under the control conditions (Fig. 2B), indicating no contamination of ectodermal lineages in Activin B induced cells.

3.1. Effect of BMP2 stimulation on monolayer primitive streak cell population

At this point hESCs could be differentiated into a mesendoderm population that expressed early and late mesoderm markers; we then endeavored to investigate the effect of BMP-2 on this mesendodermal cell population as a method of differentiation toward an osteogenic lineage. Effect of BMP-2 was tested on hESC-derived mesendodermal cells in low serum medium. We added BMP2 at day 3 of differentiation in low serum media to the cells. The cells were analyzed after an additional 3–6 days of culture for osteogenic markers. Stimulation with BMP2 for 6 days did not change the morphology of the cells, however slight increases in the expression level of osteogenic genes were observed. We found that induction with BMP2 repressed SOX17 gene expression and induced the expression of both lateral plate mesoderm marker (FOXF1) and paraxial mesoderm marker (MEOX1) in the differentiated hESCs (Kalinichenko et al., 2001). As expected, we observed an increase in gene expression of osteogenic markers such as, RUNX2, OP and collagen 1 (Col-1), however other osteogenic and chondrogenic markers such as MSX2, ALP, DLX5, SOX9 and OSX, among others, were not affected by BMP2 induction. We tested the osteogenic and adipogenic differentiation abilities of these cells by sub-culturing them on fibronectin coated culture plates in osteogenic and adipogenic media. However, these cells did not show any potentiality in differentiation toward osteogenic or adipogenic lineages (data not presented).

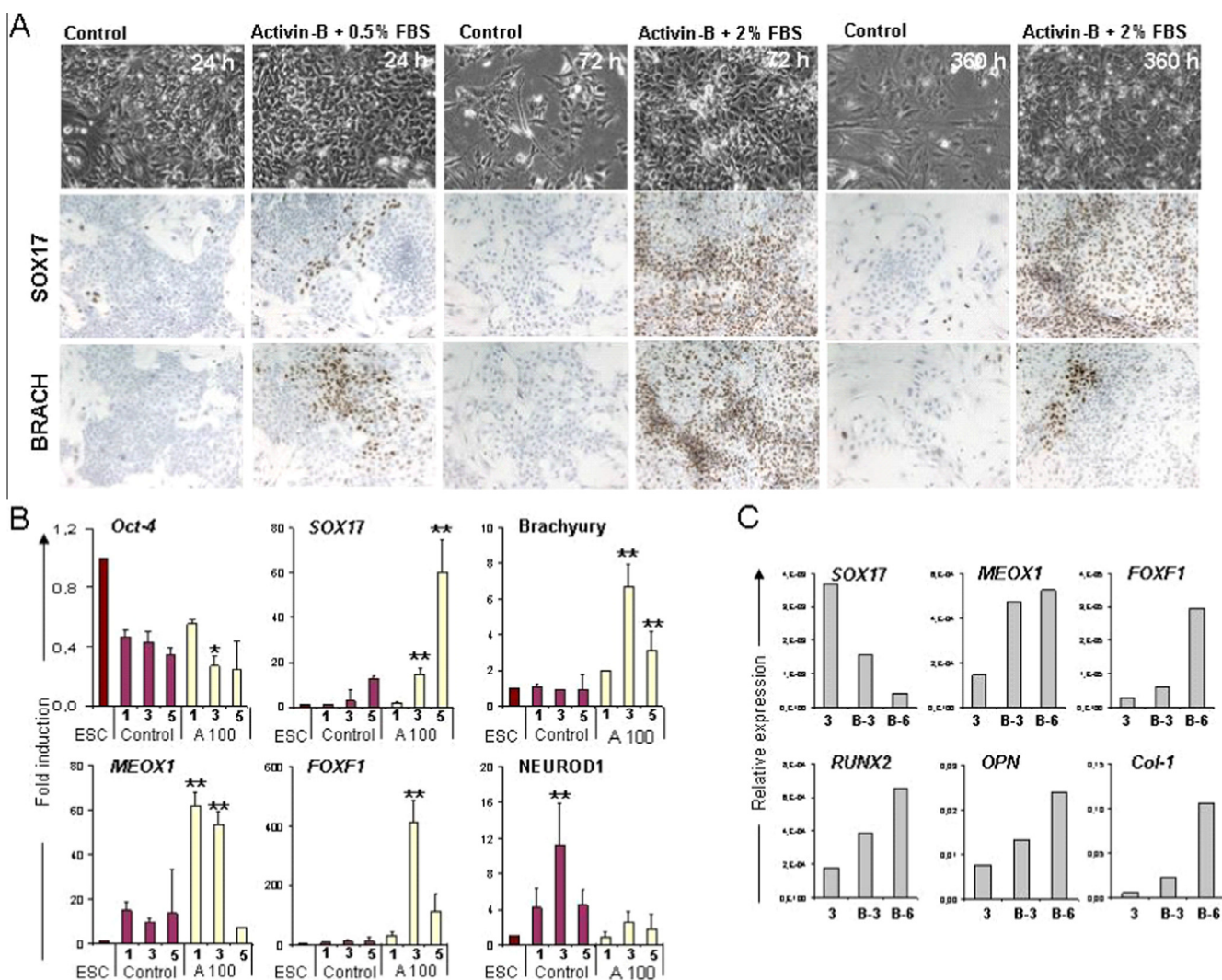


Figure 3 Analysis of direct monolayer differentiated hESCs to mesendoderm like population. (A) Upper panel showing representative light microscope images for monolayer differentiation of hESCs in the presence or absence of Activin B (100 ng/ml). Lower panel shows immunostaining of differentiated hESCs with mesendoderm marker brachyury (BRACH) endoderm marker SOX17 (original magnification 100×). (B) Total RNA was extracted and real time-PCR analysis was performed on the undifferentiated HUES9 hESCs (ESC), differentiated cells without Activin B induction (control) and differentiated in the presence of 100 ng/ml of Activin B (A 100) for 1, 3 and 5 days (1, 3, 5). Expression levels of target genes are represented as fold induction over undifferentiated hESCs, values are represented as mean \pm SD of three independent experiments. * $P < 0.05$ and ** $P < 0.005$ versus ESC. (C) Real-time PCR analysis on mesodermal and osteogenic genes in day 3 Activin B induced mesendoderm cells (3) 100 ng/ml of BMP2 induced for additional 3 days (B-3) and 6 days (B-6). The expression level of each target gene was normalized to β actin and presented as mean of three independent experiments.

4. Conclusion

Taken together these results have demonstrated a protocol for directing differentiation of hESCs through the gastrulation step of primitive streak formation into a definitive mesendodermal stage by monolayer differentiation in low serum media. In order to investigate the developmental potential of the derived mesendoderm cells differentiated by Activin B into a more mesodermal/mesenchymal direction we stimulated the cells using BMP2, which has been shown to be necessary for paraxial mesoderm development (Miura et al., 2006; Mendjan et al., 2014). Later in the development BMP2 also plays an essential role in osteogenic induction of mesenchymal

cells (Matsubara et al., 2008). BMP2 induced cells were shown to express some of the mesenchymal/osteogenic markers but failed to express the majority of markers, furthermore the morphology of the cells did not change, it maintained to be as a squamous epithelial like morphology. In conclusion, it was not possible to differentiate mesendodermal cells further into a mesenchymal phenotype in a monolayer cell culture.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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