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Neuronal induction and regional identity by co-culture of adherent human embryonic stem cells with chicken notochords and somites

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ABSTRACT The role of somites and notochords in neuroectoderm differentiation from the embryonic ectoderm and its subsequent patterning into regional compartments along rostro-caudal and dorso-ventral axes, especially in humans, remains elusive. Here, we demonstrate the co-culture effect of somites and notochords isolated from chicken embryos on the neuronal differentiation and regional identity of an adherent culture of human embryonic stem cells (hESCs). Notochord increased the efficiency and speed of neuronal induction, whereas somites had a weak neuronal inducing effect on hESCs. However, a synergistic effect was not observed when notochords and somites were used together. Moreover, in somite and notochord co-culture groups, hESCs-derived neuronal cells expressed HOXB4, OTX2, IRX3 and PAX6, indicative of dorsal hindbrain and ventral anterior identities, respectively. Our results reveal the influence of embryonic notochord and somite co-culture in providing neuronal induction as well as rostro-caudal and dorso-ventral regional identity of hESCs-derived neuronal cells. This study provides a model through which *in vivo* neuronal induction events may be imitated.

KEY WORDS: *Chicken, Co-culture, hESC, Neuronal Induction, Notochord, Somite*

Introduction

Neural induction is the process by which signals such as noggin, follistatin, and chordin that originate from surrounding tissues induce the development of the neural fate in the ectoderm (Gaulden and Reiter, 2008; Khokha *et al.*, 2005; Rashbass *et al.*, 1994; Wessely *et al.*, 2004). These molecules inhibit bone morphogenetic protein (BMP) signaling in the ectoderm and proceeds neural induction by default, in the absence of a specific neuronal inducer (Hemmati-Brivanlou., 1997; Levine and Brivanlou, 2007; Wilson and Edlund, 2001). Therefore, in this hypothesis the inductive signals from the notochord, node and somites are suggested to be unnecessary (Hemmati-Brivanlou., 1997; Munoz-Sanjuan and Brivanlou, 2002). Despite many advocates of this model, the sole role of the default model in zebra fish, chickens, and mice is questionable (Hurtado and De Robertis, 2007; Klingensmith, 1999; Shimizu, 2000; Stern, 2005; Streit, 2000; Streit *et al.*, 1998). In contrast, other reports show that neural differentiation is the result of direct neural induction by neighboring tissues (Hemmati-Brivanlou, 1994; Kuroda *et al.*, 2004; Sasai, 1995; Smith, 1992). Therefore, the role of somites and notochords in the decision of the ectoderm to acquire a neuroepithelial fate

Abbreviations used in this paper: hESC, human embryonic stem cell; N, notochord; S, somite.

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and its subsequent patterning into regional compartments along the rostro-caudal and dorso-ventral axes, especially in humans, remains elusive. Given the complexity of cellular signaling pathways *in vivo*, co-cultures are a model to assess differentiation under controlled conditions, with the advantage of being able to replicate some tissue-derived signaling (Anjomshoa *et al.*, 2009; Sagha *et al.*, 2009). Recently, we have demonstrated that co-culturing mice embryonic stem cells (ESCs) with chicken embryonic somites and notochords resulted in neuronal induction and ventral identity of differentiated neurons (Anjomshoa *et al.*, 2009; Sagha *et al.*, 2009). On the other hand, the establishment of human ESCs (hESCs) with self-renewal and pluripotency potentials provide a most amenable system to investigate these early development events in humans (Pankratz *et al.*, 2007).

Therefore, in this study we sought to examine the influence of chicken embryonic somites and notochords on the neuronal fate as well as the rostro-caudal and dorso-ventral regional identity of adherent cultured hESCs.

Results

In order to assess the effect of somites (S) and notochords (N) on human neuronal induction, two day old hESCs were cultured either alone (control group, C), in the presence of S, N, or both (S/N) which were derived from stages 9-12 chick embryos and encapsulated in alginate beads up day 9 (=2+7). Beads were subsequently removed from the plates and induced hESCs were allowed to culture in co-culture medium for an additional seven days *in vitro* (day 16= 2+7+7; Fig. 1A).

hESCs treated with N or S/N at day 16 were negative for endoderm (FOXA2, SOX17) and mesoderm

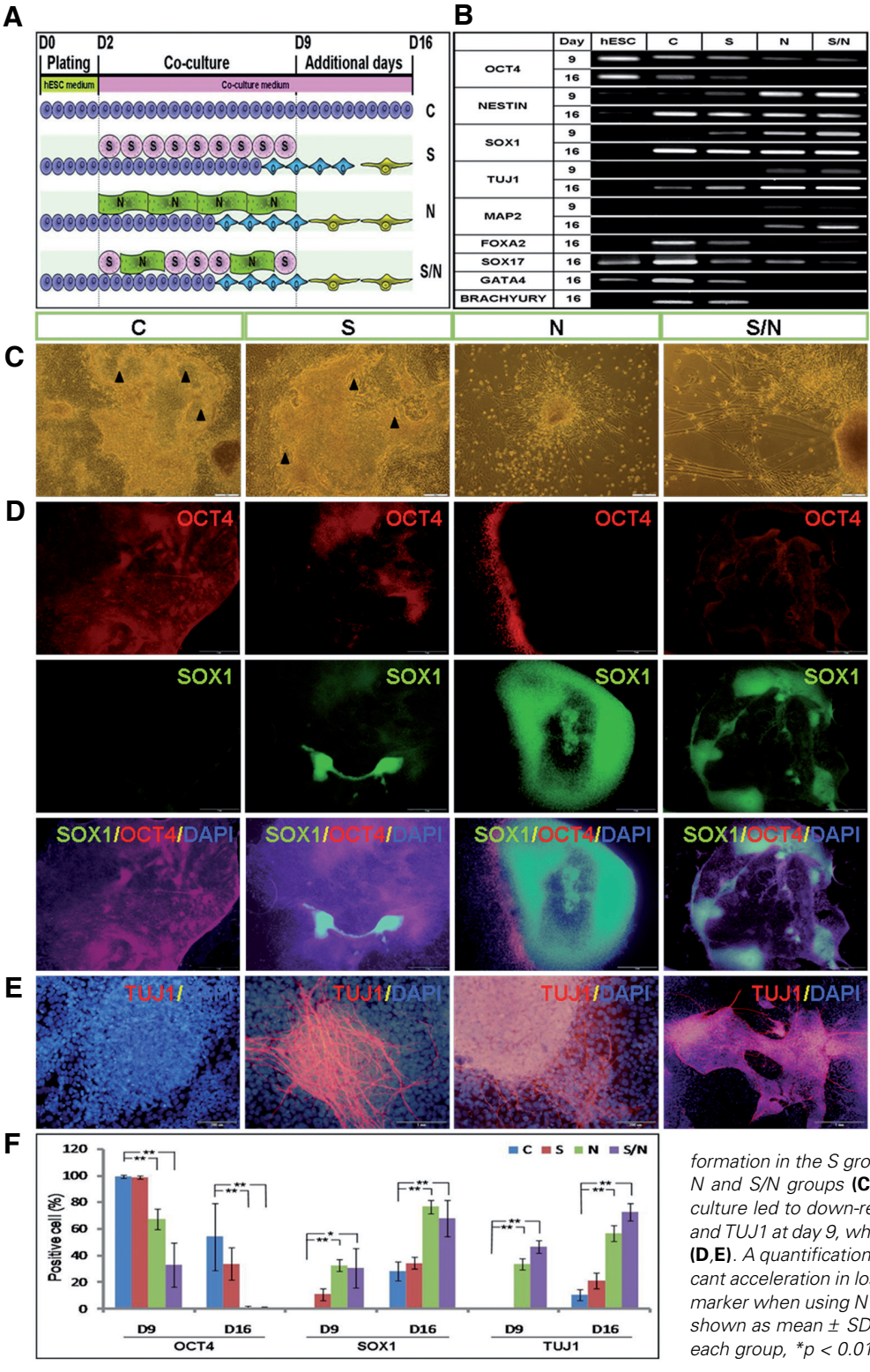

Fig. 1. Co-culture with notochord (N) and somite/notochord (S/N) accelerated neuronal induction from hESCs. *Illustration protocol of co-culture with hESCs* **(A)**. *Transcriptional analysis confirms loss of stem marker at day 9 and up-regulation of progenitor and mature neuronal markers at day 9 in N and S/N groups, the loss of endodermal markers (FoxA2 and SOX17) by day 16 and the absence of mesodermal markers (GATA4 and BRACHYURY) in both the N and S/N groups* **(B)**. *Phase contrast images of groups showed cystic formations in the C group (arrow heads), rosette formation in the S group (arrow heads) and mature neuron(s) in the N and S/N groups* **(C)**. *Neuronal induction from hESCs using co-culture led to down-regulation of OCT4 and up-regulation of SOX1 and TUJ1 at day 9, which was more intense in the N and S/N groups* **(D,E)**. *A quantification of the immune experiment confirmed significant acceleration in loss of pluripotency and expression of neuronal marker when using N and S/N compared to the control (F). Data are shown as mean ± SD (n = 8, more than 500 cells were counted in each group, *p < 0.01, **p ≤ 0.001.*

(GATA4, BRACHYURY) markers. However, these markers were expressed in the C and S groups (Fig. 1B). Moreover, the expression of OCT4, a pluripotency marker, was not detected at day 16 in the N and S/N groups. However, NESTIN (a marker of neuronal progenitor cells), SOX1 (a marker of neuronal progenitor cells), TUJ1 (a neuronal marker) and expression of MAP2 (a mature neuron marker) were detected at day 9 (Fig. 1B).

The presence of N or N/S gave rise to neuronal cells with cell body and peripheral extensions after 7 days of co-culture. However, in the C and S groups, hESCs showed only an early differentiation with rosette structures, a neuroepithelium marker with radially columnar epithelial cells at the same time (Fig. 1C).

Immunofluorescence staining of differentiated cells demonstrated down-regulation of OCT4 and up-regulation of SOX1 and TUJ1 (Figs. 1 D and E). A quantification of the immune experiment confirmed an accelerated efficient neuralization with 32.6±4.4% and 30.5±14.7% at day 9 and 76.5±4.8% and 67.9±13.6% at day 16 SOX1-positive cells and 33.5±4.2% and 46.5±4.6% at day 9 and 56.6±5.9% and 72.5±6.6% at day16 TUJ1-positive cells for N and S/N groups, respectively compared to the C and S groups ($p<0.001$, Fig. 1F). Staining for OCT4 positivity at day 16 was 54.2±25.1% and 33.7±12.1% in the C and S groups compared to negligible OCT4-positive cells in the N and N/S groups at day 16 ($p\leq0.001$, Fig. 1F) which showed a higher differentiation of hESCs in the N and S/N groups. Our experiments indicated an increase in neuronal differentiation in the presence of N only. There was no improvement in the S group when compared to the control group.

To evaluate further the influence of S, N, and S/N on the positional identity of neural progenitors, we undertook transcriptional profiling of rostro-caudal markers (OTX2, HOXB4, HOXC5 and HOXC8) and dorso-ventral markers (PAX7, IRX3, PAX6, OLIG2, NKX6.1 and NKX2.2) at day 16. These data indicated that OTX2, a rostral marker, was detectable in all groups. However, HOXB4 was only detected in the C and S groups and HOXC5 (a cervical spinal cord marker) and HOXC8 (a thoracic spinal cord marker) were not detected in all groups (Fig. 2A). The expression of OTX2 and HOXB4 was confirmed in all groups by immunofluorescence staining (Fig. 2D) and their quantification revealed higher OTX2/TUJ1 double positive cells in N and S/N groups in comparison with the C and S groups ($p<0.001$; Fig. 2D). It was interesting to note that S induced more expression of HOXB4/TUJ1 double positive cells in comparison with other groups.

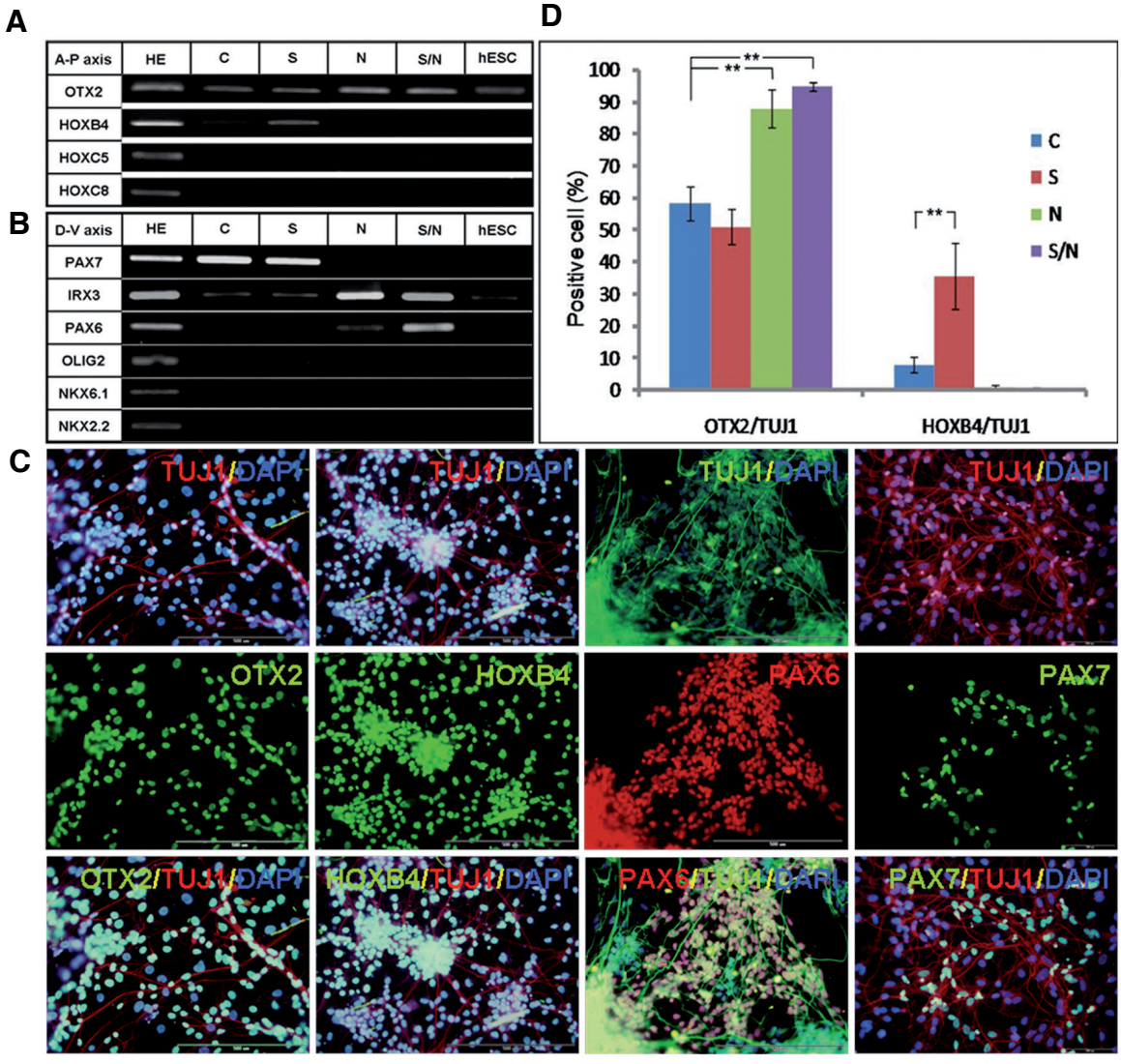

Fig. 2. Influence of somite (S) and N (notochord) on neural progenitors regional identity. *RT-PCR analysis of rostro-caudal and dorso-ventral axes' genes revealed OTX2 expression in all groups, HOXB4 in the S group, PAX7 in the C and S groups, and ventral markers, IRX3 and PAX6, in the N and S/N groups* **(A,B)**. *Immunostaining for OTX2 in the C group, HOXB4 and PAX7 in the S group, and PAX6 in the S/N group confirmed the RT-PCR results* **(C)**. *A quantification of the immune experiment showed significant expression of the rostral marker, OTX2, in the N and S/N groups, and hindbrain marker, HOXB4, in the S group in relation to TUJ1 positive cells when compared with the C group (n = 8, more than 500 cells were counted in each group, $**p\leq0.001$* **(D)**.

Therefore, it seems that S and N make hindbrain and anterior identities for neuronal cells, respectively. Assessment of dorso-ventral axis markers using RT-PCR revealed that the N and S/N groups induced the expression of ventral markers IRX3 and PAX6, but not other related ventral markers (OLIG2, NKX6.1 and NKX2.2; Fig. 2B). In contrast, the dorsal marker PAX7 was expressed in both the S and C groups (Fig. 2B). The expression of PAX7 and PAX6 was also detected at the protein level by immunofluorescence staining in the S and S/N groups, respectively (Fig. 2C).

Discussion

The current study examined the co-culture effect of encapsulated chicken embryonic notochords and somites on neurogenesis as well as rostro-caudal and dorso-ventral regional identity of adherent hESCs. Microencapsulation of somites and notochords into alginate were performed to generate a co-culture system which has been shown to be a good approach to study the interaction between different tissues (Sugie *et al*., 2005). In this way, somites and notochords were kept separated from hESCs and also allowed the molecules produced by the encapsulated cells to diffuse out into the environment (Erstesvag and Valla, 1998; Smidsrod and Skjak-Braek, 1990).

Our co-culture system with S gave rise to a weak neuronal differentiation as indicated by expression of SOX1 and TUJ1. We previously showed that chicken somites induced rosette structures and weakly enhanced neuronal differentiation from mice ESCs (Sagha *et al*., 2009) which was in accordance with the"co-culture of somites and ectoderm in *Xenopus* (Jones and Woodland, 1989). On the other hand, in the S co-culture group, hESC-derived neuronal cells showed HOXB4 and PAX7 expression and dorsal hindbrain identity which was in accordance with previous *in vivo* reports (Chapman, 2004; Gould *et al*., 1998; Itasaki *et al*., 1996; Wilson, 2005). Similar expression of HOXB4 and PAX7 was reported in mice ESC-derived neuronal cells following co-culture with chicken somites (Sagha *et al*., 2009). However, such fate was not observed in the S/N group's neuronal cells which possibly suggested that the notochord has an inhibitory effect on expression of HOXB4 and PAX7 by somites. The mechanism of somites on neuronal differentiation and possibly caudalization is not clear, however there are several reports indicating that somites secret RA and BMPs antagonists (Deschamps and van Nes, 2005, Diez del Corral and Storey, 2004; Dupe and Lumsden, 2001; Gould *et al*., 1998; Itasaki *et al*., 1996; Lewis, 2006; Liem *et al*., 2000; Maden, 2006). We have previously demonstrated the secretion of retinoids by chicken somites (Sagha *et al*., 2009), although the levels of all-*trans*-RA by the somites is low (10^{-9}-10^{-10} M) (Maden *et al*., 1998). It seems that this neurogenic effect and hindbrain induction of somites may be a reflection of these signaling molecules however; its mechanism remains to be elucidated.

Moreover, the N group resulted in earlier and higher expression of neuronal markers SOX1, TUJ1 and MAP2 when compared to the C and S groups. Traditionally, the notochord has been believed to be involved in neuronal induction (Harland, 2000; Le Douarin, 2001) while our previous research showed that only the N group did not induce neuronal differentiation of mice ESC-derived embryoid bodies (EBs) (Anjomshoa *et al*., 2009). This discrepancy is likely related to the adherent culture implemented in this study instead of a suspension culture by EB formation which prevent the formation of neurectoderm by endogenous Wnt signals (ten Berge *et al*., 2008) or presence of fetal calf serum (5%) which contained BMPs or species difference.

The neural inductive role for notochord is not far from expectation, since notochord secrets Shh and BMPs antagonists such as noggin, chordin, follistatin, and flik in the chicken among other vertebrate species (Rolf W. Stottmann, 2006) which have always been involved in neural induction *in vivo* and *in vitro.* Moreover, hESC-derived neuronal cells in the N group had a ventral anterior identity with OTX2, IRX3 and PAX6 expression, which suggests that the N influences neuronal specification in both the rostro-caudal and dorso-ventral axes. It has been reported that the competent ectoderm of *Xenopus* embryos (stage 9, late blastula) when wrapped around the anterior notochord of stage 12.5 expressed higher En2, an early anterior neuroectoderm marker, like OTX2 that expresses in this domain compared to the ectoderm which wrapped posterior notochord (Brivanlou and Harland, 1989; Hemmati-Brivanlou *et al*., 1990). The creation of distinct classes of neurons within the dorso-ventral axis of the developing neuronal tube depends on differential exposure to BMP, Shh and RA from surrounding tissues and this exposure must be carried out at an appropriate time and duration (Ericson, 2001; Jessell, 2000). The reason for the lack of other specific gene expressions may arise from limited co-culture time and the lack of a suitable time window for the neuronal regional identity effect of secreted molecules during co-culture with hESCs or other unknown mechanisms by which these effects are induced.

In conclusion, the results of the present study suggest that the N group has a strong neurogenic effect on adherent hESCs. In addition, S induces hindbrain identity in the hESC-derived neuronal cells while the N group maintained their anterior identity of hESC-derived neuronal cells. The mechanisms by which these effects are induced remain to be elucidated.

Materials and Methods

Preparation of encapsulated alginate bead somites and notochords

Chick eggs were provided from commercial sources and incubated in a humidified atmosphere at 38°C. Embryos of Hamburger and Hamilton stages 9-12 were used for our experiments (Fig. 1A). Chick embryos were isolated from the yolk surface and transferred into Leibovitz's (L15) medium (Invitrogen, 41300-021). Then, embryos were placed in new L15 medium that contained dispase (1 mg/ml, Invitrogen, 17105-041) for 3–5 min with the purpose of slackening the chick embryo tissues. The enzyme was removed and embryos were washed with L15 medium supplemented with 5% fetal calf serum (FCS, Invitrogen, 16141-079) for 15 min. Subsequently, embryos were transferred into the clod L15 medium without FCS. Somites and notochords were subsequently isolated from embryos under a dissecting microscope and transferred to co-culturing medium. Eventually, 192 somites and 80 notochord pieces (12 somites and 5 notochords/colony) were encapsulated into alginate beads (8 beads) for 16 hESCs colonies in 24 wells (replicate ≥12). Alginate beads were prepared according to previous references (Anjomshoa *et al*., 2009; Sagha *et al*., 2009).

Culturing hESCs

The feeder free hESC line, Royan H5 (Baharvand *et al*., 2006) at passages 50-70 was used for these experiments. For hESC maintenance, differentiated cells were removed by gently pipetting. After collagenase IV (1 mg/ml, Invitrogen, 17104-019) treatment the colonies were

mechanically dissected into small pieces and replated on Matrigel-coated dishes with hESC medium containing DMEM/F12 medium (Invitrogen, 21331-020) supplemented with 20% knock-out serum replacement (KOSR, Invitrogen, 10828-028), 2 mM L-glutamine (L-glu, Invitrogen, 25030-024), 0.1 mM β–mercaptoethanol (BME, Sigma-Aldrich, M7522), 1% nonessential amino acids (NEAAs, Invitrogen. 11140-035), 100 units/ml penicillin and 100 μg/ml streptomycin (pen/strep, Invitrogen, 15070-063), 1% insulin/transferring/sodium selenite (ITS, Invitrogen, 41400-045) and 100 ng/ml basic-fibroblast growth factor (bFGF, Royan Institute). The medium was changed daily. Cells were cultured in 5% CO_2 at 95% humidity and were further passaged on a weekly basis.

Co-culture with somites and notochords

hESC colonies, two days after plating in hESC medium on Matrigel, were co-cultured by somites, notochords, and/or both in co-culture medium that included DMEM/F12 supplemented with 5% KOSR, pen/strep, 1% NEAAs, 2 mM L-glu, 1% ITS, 0.1 mM BME, and 2% B27 (Invitrogen, 17502-044). After seven days, the beads that contained somites and notochords were removed and colonies were allowed to undergo further differentiation for an additional seven days in co-culture medium. Half of the medium was renewed every other day.

Immunoflourescence staining

Differentiated cells were washed in PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, P6148) in phosphate buffered saline (PBS) for 30 min. Fixed cells were washed twice with PBS before staining. Permeabilization was carried out by 0.2% Triton X-100 in PBS for 1 hour. Primary antibodies were applied in blocking buffer [10% goat serum and 1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, A3311) in PBS] for 1 hour at 37˚C or overnight at room temperature and washed three times in blocking buffer before the addition of a secondary antibody. Secondary antibodies were diluted in blocking buffer and applied to cells for 2 hours at room temperature. After two washes in PBS, 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D8417) was applied for 3 min for nuclear counterstaining and cells were observed under fluorescence microscope (Olympus, BX51, Japan). Quantification was estimated by the percentage of positive cells in comparison to the total cells as indicated by DAPI in the fields. For negative controls, primary antibodies were omitted and the same staining procedure was followed. Primary and secondary antibodies are presented in Supplementary Table 1.

Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from cultured cells using the QIAprep Spin Miniprep kit (QIAGEN, 27106) according to the manufacturer's protocol. Prior to reverse transcription (RT), RNA samples were digested with DNase I (Fermentas, EN0521) to remove contaminating genomic DNA. Standard RT was performed using 2 μg total RNA, oligo (dT) 18 and the RevertAidTM H Minus First Strand cDNA Synthesis kit (Fermentas, K1622) according to the manufacturer's instructions. Primer sequences, annealing temperature(s), cycles and the lengths of amplified products are shown in Supplementary Table 2. Amplification conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 45 sec, extension for 45 sec at 72°C and a final polymerization at 72°C for 10 min. PCRs were performed in triplicate. PCR products were analyzed by gel electrophoresis on 1.7 or 2% agarose and stained with ethidium bromide (10 μg/ml), visualized and photographed on a UV transilluminator (Uvidoc, UK). The CNS RNA of a 20 week old human embryo was used as a positive control for RT-PCR analysis.

Statistical analyses

Quantification of immune and PCR experiments was performed based on eight and three replications, respectively. Data of quantification of the immune staining were expressed as mean ± SD (standard deviation). One-way ANOVA followed by Tukey's post hoc multiple group comparison test was used to analyze group differences of the data collected from immunofluorescence staining. A difference between groups was considered as statistically significant if $p<0.05$.

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