Activin A Suppresses Induced Formation of Neuroectoderm in Colonies of Parthenogenetic Stem Cells *In Vitro*

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We studied the effect of recombinant human activin A on induced neuroectoderm formation in colonies of human parthenogenetic SC in the absence of feeder cells. It was found that pretreatment of human parthenogenetic SC with activin A suppressed subsequent neural induction. Activin A in a concentration of 10 ng/ml significantly decreased transcriptional activity of genes required for neuroectoderm formation. At the same time, activin A in a concentration of 20 ng/ml increased the expression of pluripotency genes and completely inhibited the formation of structures *in vitro* reproducing the neural tube of the developing embryo. These findings attest to prolonged effect of activin A as an inhibitor of neuroectodermal differentiation.

Key Words: *activin A; neural induction; parthenogenetic stem cells*

Human parthenogenetic stem cells (HPSC) were first isolated by E. S. Revasova with co-workers from the inner cell mass of blastocysts developing without male gamete as a result of artificial activation of non-fertilized oocytes [5,6]. By their properties, including unlimited proliferation capacity and pluripotency, HPSC are similar to human embryonic cells (HESC). Isolation of HPSC is not related to fertilization followed by destruction of a human embryo, which makes them more acceptable from ethical point of view for potential therapeutic application. The genome of HPSC is formed on the basis of only maternal chromosome set and depending of the derivation method can be completely homozygous or homozygous by the main HLA haplotypes, which makes HPSC immunologically universal for many recipients [5]. Thus, HPSC are an attractive source of differentiated and somatic SC for research and therapeutic purposes.

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Culturing of HPSC implies maintenance of pluripotency and suppression of differentiation with exogenous growth factors; LIF and/or bFGF are more widely used for this purpose. Activin A, a member of transforming growth factor-β superfamily, can maintain pluripotency of HESC in the absence of feeder cells [1], which allows using this factor in cases when culturing with feeder cells is undesirable.

Activin A prevents retinoid acid-induced neural differentiation of mouse embryonic carcinoma cells [3]. On the contrary, inhibition of Activin/Nodal signal pathway promotes neural induction in HESC colonies [3,8]. It was found that activin A inhibits expression of *PAX6* gene essential for the formation of neuroectoderm during *in vitro* differentiation of isolated neural plate in chick embryo [4]. Thus, exogenous activin A also specifically suppresses the formation of neuroectoderm by maintaining SC pluripotency.

It remains unclear whether activin A pretreatment of SC can affect neural induction after its withdrawal.

Here we studied the effect of pretreatment of HPSC with recombinant human activin A on subsequent formation of the neuroectoderm and expression of specific regulatory and marker genes.

MATERIALS AND METHODS

HPSC (strains phESC-1, phESC-3 [6] and hpSC-Hhom-4 [5]) were cultured on mitomycin C-treated mouse embryo fibroblasts (Millipore) as a feeder in a medium containing KDMEM/F12 medium (Invitrogen), 15% KSR (knockout serum replacement, Invitrogen), 2 mM L-glutamine (GlutaMAX-I, Invitrogen), 0.1 mM nonessential amino acids MEM (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin/amphotericin B (MP Biomedicals), and bFGF in a final concentration of 5 ng/ml (Peprotech). The cells were subcultured 1:4 or 1:6 every 5-7 days using dispase (Invitrogen). Activin A in concentrations of 10 and 20 ng/ml was used in experimental groups during 5 passages on the feeder; control HPSC cultures were not treated with activin A.

For neural induction, HPSC were seeded on 60-mm plastic Petri dishes coated with adhesion matrix CELLstart (Invitrogen) and cultured for 4 days in the same medium with recombinant human activin A (R&D Systems) in concentrations of 10 and 20 ng/ml in the corresponding experimental groups and without this factor in the control group. On day 5, the medium was replaced with a neuroinductive medium of the following composition: DMEM/F12 medium, neutralizing agent N2 (Invitrogen), 0.1 mM nonessential amino acids MEM (Invitrogen), 2 mM L-glutamine (GlutaMAX-I, Invitrogen), 1% penicillin/streptomycin/amphotericin B (MP Biomedicals), and 20 ng/ml bFGF (Peprotech). Activin A was not added to the neuroinductive medium.

Total RNA was isolated using robotized QIAsymphony system (Qiagen) according to manufacturer's instructions. The reverse transcription reaction was performed with 100-500 ng total RNA using iScript cDNA synthesis kit (Biorad). Real-time PCR was performed on Rotor-Gene Q cycler (Qiagen) using a Quantitest SYBR Green master mix kit (Qiagen) repeatedly for each cDNA sample using commercial QuantiTect Primer Assay primers (Qiagen) for the following genes: *MS1* (QT00025389), *NES* (QT00235781), *OLIG2* (QT01156526), *PAX6* (QT00071169), *POU5F1/OCT4* (QT00210840), *NANOG* (QT01844808), *SOX1* (QT01008714), *SOX2* (QT00237601), *SOX3* (QT00212212), *PPIG* (QT01676927). The relative calculations were performed using a standard curve; quantitative values were standardized by the expression of house-keeping gene PPIG (cyclophilin G). The differences in gene transcription activity at different activin concentration were evaluated using Mann–Whitney *U* test for $n_1,n_2 \leq 5$.

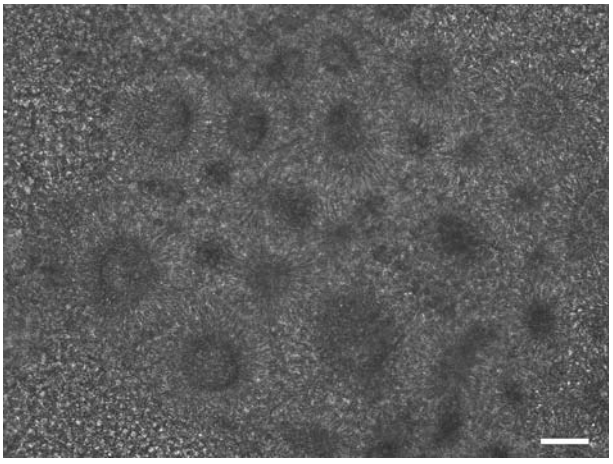

Fig. 1. Rosettes of neuroectodermal cells obtained from HPSC in the adhesion model. Phase contrast. Scale: 200 μ.

RESULTS

We used adhesion model for neural induction and obtaining neuroectoderm [7]. In contrast to original protocol, we performed neural induction in colonies of HPSC grown on CELLstart matrix without feeder cells.

Intensive formation of neuroectoderm in the form of typical structures, rosettes of pseudostratified epithelium with a central clarified space, was observed over 7 days in HPSC colonies of the control group grown on neuroinduction medium (Fig. 1). Pretreatment with activin A in a concentration of 10 ng/ml led to the formation of solitary irregularly-shaped rosettes. No rosettes were formed in HPSC colonies growth in the presence of 20 mg/ml activin A.

Activity of *SOXB1* group genes and *PAX6* gene essential for the formation of neuroectoderm was considerably reduced at the stage of neural induction after pretreatment with 10 ng/ml activin A, compared to pretreatment with 20 ng/ml activin A (empirical *U* values are significant at $p \leq 0.05$). Expression of neuroectodermal marker genes *NES* (*Nestin*) and *MS1* (*Musashi-1*) was also the lowest in this group (Fig. 2). Despite this fact, the formation of neuroectodermal rosettes was not completely suppressed after treatment with 10 ng/ml activin A. At the same time, activin A in a concentration of 20 ng/ml increased the expression of pluripotency genes *OCT4* (POU5F1) and *NANOG* and completely inhibited the formation of rosettes against the background neural marker expression. This was accompanied by a decrease in the expression of transcription factor *OLIG2* essential for the formation of the neurogenic domain of the nerve tube.

Activity of *SOX2*, *SOX3*, *NES*, *OCT4*, *MS1*, and *NANOG* genes increased in HPSC cultures grown in the presence of activin A. Activity of *NANOG* continued to increase during HPSC culturing without feeder

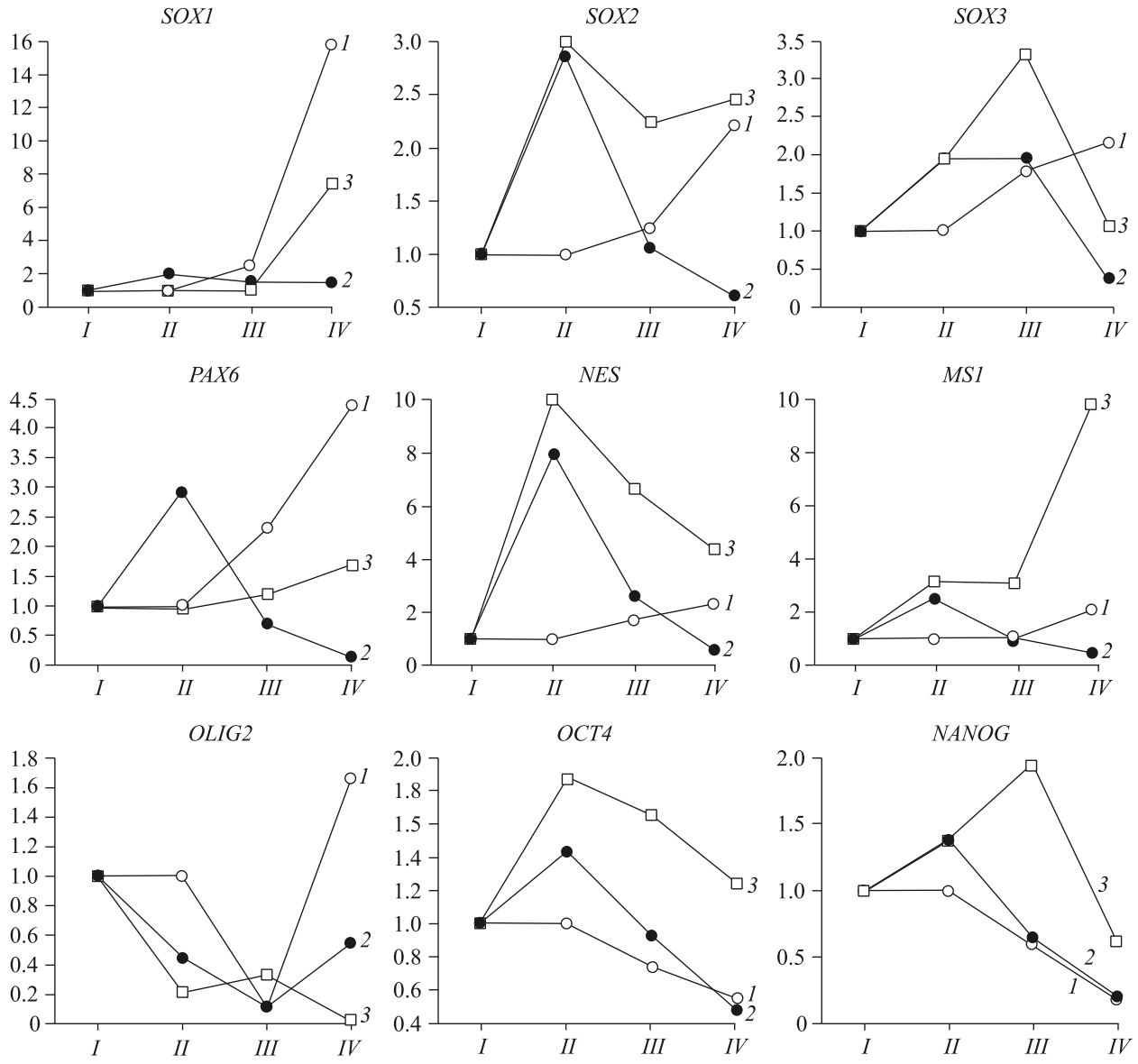

Fig. 2. Changes in gene transcription activity in HPSC after activin A treatment. I: transcription activity of the studied genes in HPSC colonies on feeder were taken as 1, II: HPSC grown on feeder in the presence of activin A; III: HPSC grown on CELLstart matrix in the presence of activin A; IV: neural induction in the absence of activin A in concentrations 0 (*1*), 10 (*2*), and 20 ng/ml (*3*).

and decreased after withdrawal during neuronal induction. Activity of *PAX6* increased at activin A concentration of 10 ng/ml and decreased during HPSC culturing without feeder and during neural induction. At the same time, activin A concentration of 20 ng/ml considerably increased MS1 activity. These findings suggest that the effects of activin A on gene expression in HPSC differ under conditions of HPSC culturing on feeder and CELLstart matrix, while aftereffects of activin A on the gene expression during neural induction depends on concentration of this factor during pretreatment.

Thus, our experiments demonstrated prolonged inhibitory effect of activin A on neuroectoderm formation. The use of activin A for maintaining pluripotency and improving the growth of SC colonies can be undesirable for subsequent obtaining neural derivatives.

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