Application note

Title: Biological activity of Activin A assessed via induction of endodermal and cardiac lineage protein markers expression from human induced pluripotent stem cells.

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Background:

Activin A/ Nodal protein (also known as Inhibin A, encoded by INHBA gene) is a 26 kDa disulfide-linked homodimer of two Inhibin beta A chains, each containing 116 amino acid residues. Activin A homodimer has a major role in cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune response, wound repair, and endocrine function. Activin A is a member of the TGF-beta (transforming growth factor-beta) superfamily of proteins, activating Alk4 and Alk7 receptor-like kinases, followed by Smad2/3 activation, formation of dimer complex with Smad4, nuclear translocation and subsequent activation or inhibition of nuclear factors-mediated gene transcription in cells. Initially, Activin was discovered as a gonadal protein that induced follicle-stimulating hormone (FSH) release. Since then, it has been linked to other signaling and regulatory pathways and has been found to be expressed in many different cell types at nearly all stages of development (Ling et al., 1986; Vale et al., 1986). Activin A's activity has been established as a critical regulator during embryonic development, controlling the expression of pluripotency factors and differentiation of embryonic germ layers (Pauklin and Vallier, 2015). Various key roles played by Activin in developing and growing tissues also directly relate to tumorigenic processes and maintenance of adult tissue.

Activin A plays a direct role in the initiation of cardiac and endocrine cell development *in vivo* (mesoendodermal lineage), while inhibiting ectodermal differentiation, and was shown to be highly effective during the induction of the *in vitro* differentiation of pluripotent stem cells (embryonic and induced) toward these lineages (Pauklin and Vallier, 2015). So far, no small chemical compound or short synthetic peptide has been successful replacing the full spectrum of Activin A biological activity in tissue culture and differentiating cells (Firestone and Chen, 2010). During production of recombinant Activin A, its biological activity is usually confirmed by the induction of cytotoxicity on MPC-11 mouse myeloma cells.

Study design

The presence of Activin A in a multitude of critical signaling pathways makes it useful in functional assays for cell differentiation. Here we set to use Activin A protein in two differentiation protocols (endoderm and cardiomyocytes differentiation from pluripotent stem cells) for the induction of expression definitive endoderm marker SOX-17 and cardiac lineage marker MYH. Expression of both markers is assessed by FACS of samples collected from planar cultures upon completion of the protocol stage.

Methods

Cell culture and differentiation

Two human induced pluripotent lines (iPSCs) ATCC-HYS0103 and KYOU-DXR0109B (ATCC, Manassas, VA) were grown on hESC-qualified Matrix Matrigel-coated (1:32 dilution in DMEM/F12 media, Corning) flasks in mTesr-1 media (StemCell Technologies, Vancouver, Canada). Cells were passaged using Accutase or ReLeSR methods and seeded in Matrigel-coated 6-well plates (Corning, Corning, NY) at 500,000 cells/well, using 10 µM Rock inhibitor compound (Y27632, Tocris, Bristol, UK) in mTesr-1 for the first 24h of seeding, and for additional 48h in culture in mTesr-1 only. For definitive endoderm (DE) differentiation we used a modified first stage of the protocol described here (Russ et al., 2015) and for the cardiac lineage (CD) differentiation we used a protocol as described in (Hudson et al., 2012). For DE induction cells were washed twice with DPBS without Ca^{2+}/Mg^{2+} and RPMI1640-GlutaMAX (Gibco, ThermoFisher, Waltham, MA) media was added, containing 100ng/ml Activin A (Cellaria Biosciences, Natick, MA), 1:5000 ITS supplement (Gibco), 3 µM CHIR compound (Wnt signaling inhibitor, Tocris) and 0.2% FBS (Corning) for 24h in 37^0C in 5% CO_2 incubators. Then, the media was replaced with RPMI1640-GlutaMAX (Gibco), containing 100ng/ml Activin A (Cellaria Biosciences), 1:2000 ITS supplement (Gibco) and 0.2% FBS (Corning) for the next 24 hours. We used iPSCs differentiated with STEMdiff™ Trilineage Differentiation Kit (StemCell Technologies) as control samples for DE induction.

For CD differentiation protocol, cells were washed as described above with DPBS and media was added with a following formulation, RPMI1640-GlutaMAX with 6ng/ml Activin A (Cellaria Biosciences), 20 ng/ml BMP-4 (R&D Systems), and 1% penicillin/streptomycin with 1:50 dilution of B-27 supplement (both Gibco) for 72 hours. After that, media formulation was changed to RPMI1640-GlutaMAX (Gibco), 5 µM IWP-4 (Wnt inhibitor, Tocris) and 1% penicillin/streptomycin with 1:50 dilution of B-27 supplement for 12 days incubation with media changes every 48 hours.

FACS staining and analysis

After completion of the differentiation protocol, cells were washed twice with PBS without Ca^{2+}/Mg^{2+}, collected with TryplE Express and fixed with 4% paraformaldehyde in DPBS without Ca^{2+}/Mg^{2+}. For intracellular SOX-17 (DE protein marker) detection, rabbit anti-SOX17 antibody (Rockland, Limerick, PA) was diluted to 2.5 µg/ml concentration in blocking buffer solution (DPBS, 5%FBS and 0.1% Saponin) and detected using a goat anti-rabbit Alexa488 secondary IgG

diluted to 2 µg/ml (Abcam, Cambridge, MA). For MYH7 detection (Beta (β)-myosin heavy chain protein, cardiac lineage marker) we used monoclonal antibody hybridoma concentrate (A4.951, DSHB, Iowa City, Iowa) diluted 1:300 and secondary IgG goat anti-mouse Alexa488 (Abcam, Cambridge, MA) as detection antibody. All samples were analyzed with Accuri C6 flow cytometer (BD Biosciences) and the Accuri C6 software. Cells stained only with secondary IgGs (2' Ab only) were used as gating controls for non-specific labeling threshold.

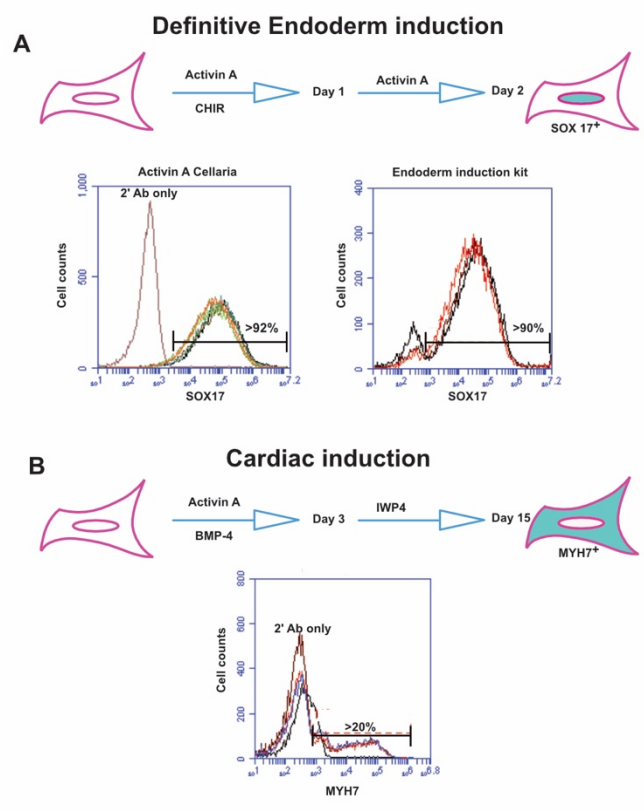

Figure 1. **A.** SOX17 expression. iPSC lines (n=6 for each line) were differentiated as described. SOX17 expression was measured via FACS and gating was done using secondary IgG (2' Ab). SOX 17 expression in iPSCs differentiated with Activin A protein was similar to one in cells differentiated with STEMdiff™ Trilineage Differentiation Kit (above 90% SOX17-positive cells in both samples, representative FACS plots and gating are shown). **B.** MYH7 expression. iPSC lines (n=3 for each line) were differentiated as described. Only iPSC line ATCC-HYS0103 remained attached and viable during the protocol, and was analyzed. Expression of MYH7 protein in ATCC-HYS0103-derived cardiac cells was above 20%, representative FACS plots and gating are shown. Each line represents one sample from differentiated iPSCs, no difference was observed between the two iPSC lines used.

Results and Discussion

FACS results from the study are shown in Figure 1.

Our aim was to confirm the activity of Activin A protein in a functional assay for pluripotent stem cells differentiation. We chose two differentiation protocols from well-established and highly referenced publications, focusing on stages where Activin A function is required, and a lineage-specific protein marker had previously been identified to measure the activation of Activin A-dependent signaling pathways and cell development events. We were able to show Cellaria's Activin A functions in endoderm and cardiac differentiation protocols, and obtained expected increases for SOX17 and MYH7 expression in our stem cell cultures.

References

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