Small molecule regulators of postnatal Nkx2.5 cardiomyoblast proliferation and differentiation

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Abstract

While recent data have supported the capacity for a neonatal heart to undergo cardiomyogenesis, it is unclear whether these new cardiomyocytes arise from an immature cardiomyoblast population or from the division of mature cardiomyocytes. By following the expression of enhanced Green Fluorescent Protein (eGFP) in an Nkx2.5 enhancer-eGFP transgenic mice, we have identified a population of immature cells that can undergo cardiomyogenic as well as smooth muscle cell differentiation in the neonatal heart. Here, we examined growth factors and small molecule regulators that potentially regulate the proliferation and cardiomyogenic *versus* smooth muscle cell differentiation of neonatal Nkx2.5-GFP+ cells *in vitro*. We found that A83-01 (A83), an inhibitor of TGF-βRI, was able to induce an expansion of neonatal Nkx2.5-eGFP+ cells. In addition, the ability of A83 to expand eGFP+ cells in culture was dependent on signalling from the mitogen-activated protein kinase kinase (MEK) as treatment with a MEK inhibitor, PD0325901, abolished this effect. On the other hand, activation of neonatal Nkx2.5-eGFP+ cells with TGF-β1, but not activin A nor BMP2, led to smooth muscle cell differentiation, an effect that can be reversed by treatment with A83. In summary, small molecule inhibition of TGF-β signalling may be a promising strategy to induce the expansion of a rare population of postnatal cardiomyoblasts.

Keywords: Nkx2.5 ● cardiomyoblast ● TGF-β

Introduction to postnatal cardiac progenitor cells

Recent studies have identified a number of stem/progenitor cell-like populations in the adult heart [1]. Despite their presence, the capacity for cardiomyocytes to undergo spontaneous renewal occurs gradually, decreasing from 1% annually at the age of 25 to 0.45% at the age of 75. This results in approximately 50% of the cardiomyocytes in the adult heart to be renewed during a normal life span [2]. The mechanisms that can lead to new cardiomyocyte generation in the postnatal human heart include the expansion and subsequent differentiation of residual cardiac progenitor cells (CPCs) or by cardiomyocyte division. Alternatively, the transplanta-

tion of ES/iPS cell-derived cardiac cells may serve such purpose as well.

During development, the expression of Nkx2.5 represents the commitment of mesodermal precursors to cardiac cells [3, 4]. Using Nkx2.5 enhancer-eGFP transgenic reporter mice to label Nkx2.5^{+} cells with an embryonic phenotype, we have previously shown that embryonic Nkx2.5[+] CPCs are capable of bi-potential differentiation into cardiomyocytes and smooth muscle cells [4]. We have recently found that eGFP^{+} cells from this reporter mouse can be identified in the hearts of neonatal and young adult mice where its abundance decreases from 5% (of non-myocytes) at 1–2 weeks of age to less than 0.3% at 8 weeks of age (unpublished data). While the existence of postnatal Nkx2.5^{+} cells with an embryonic phenotype raises the possibility that they may participate in cardiogenesis in an injured heart, thus far, no information is available regarding the signalling pathways that regulate the proliferation and/or differentiation of these cells.

Transforming growth factor-β (TGF-β) signalling plays an important role in regulating cell proliferation, differentiation, recognition, migration and apoptosis during development as well as in the maintenance of homeostasis in pathological contexts during adult life in species ranging from flies and worms to mammals [5–10]. Transforming growth factor-β family members include various isoforms of TGF-β, bone morphogenetic proteins (BMPs), activin, nodal, anti-Mullerian hormone and other structurally related factors [11]. These factors are highly conserved from vertebrates down to insects and nematodes. Transforming growth factor-β family contains two sub-families, the TGF-β/activin/nodal subfamily and the BMP/growth and differentiation factor (GDF)/Mullerian inhibiting substance (MIS) sub-family [12]. Signalling by TGF-β through Alk5 and subsequent Smad2/3 phosphorylation leads to inhibition of EC proliferation and migration [13]. Given these multi-faceted roles of TGF-β, we examined whether small molecule regulators of TGF-β signalling can affect the self-renewal and/or differentiation of Nkx2.5-eGFP^{+} cells in the postnatal heart.

Methods to examine postnatal CPCs with small molecules

Transgenic mice

Nkx2.5 enhancer-eGFP transgenic mice [4] were used to isolate cardiac Nkx2.5-GFP^{+} cells for cell culture *in vitro* and to assess the dose–response effect of drug on the expansion of cardiac Nkx2.5-GFP^{+} cells *in vivo*. All animal experiments have been approved by the Subcommittee for Research Animal Care (SRAC) at Massachusetts General Hospital.

Cell culture

The cardiac Nkx2.5-GFP^{+} cells were isolated by Fluorescence Activated Cell Sorting (FACS) purification of collagenase -A and -B (10 mg/ml; Roche Diagnostics, Indianapolis, IN, USA), which treated

neonatal hearts at the age of 2–3 weeks. See additional details in Supporting Information.

Measurement of cell proliferation

The effect of small molecules on Nkx2.5-GFP[+] cell proliferation was assessed by CYQUANT® assay (Invitrogen, Carlsbad, CA, USA). The percentage of the net change in the fluorescence intensity between vehicle control and drug group within the same experiment was calculated to establish the dose–response curve and to assess the EC$_{50}$. This was further analysed by the Hill's equation [$R/R_{max} = K^n/(K^n + X^n)$], where X represents the concentration of the small molecule and n is the Hill's coefficient.

Flow cytometry

The percentage of eGFP[+] cell within total cells from cultured cells was analysed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Propidium iodide was added to exclude dead cells.

Immunofluorescence

For immunostaining of culture cells, cells were fixed by 4% paraformaldehyde for 10 min. at room temperature, and then washed by PBS. Cells were then immunostained with antibodies against cardiac troponin T (Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA) and smooth muscle myosin heavy chain (smMHC; Biomedical Technologies, Inc. Stoughton, MA, USA). Cell nuclei were counterstained with DAPI (4´,6-diamidino-2-phenylindole, dihydrochloride; Invitrogen).

Chemical reagents

A83-01 (Alk4, 5 and 7 inhibitor) and PD0325901 (MEK inhibitor) were purchased from Stemgent (San Diego, CA, USA). Recombinant human TGF-β1, human/mouse/rat activin A and human BMP2 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Results and discussion of small molecule effects on CPCs

Modulation of TGF-β but not activin A or BMP2 signalling affects smooth muscle cell differentiation of neonatal Nkx2.5[+] cardiomyoblasts

We have previously shown that embryonic Nkx2.5^{+} cells are bi-potent for cardiomyocyte and smooth muscle cell differentiation [4]. However, FACS-purified Nkx2.5-eGFP^{+} cells from the neonatal heart appear to lack the ability to differentiate spontaneously into

cardiomyocytes while retaining only limited capacity for smooth muscle cell differentiation (Fig. 1A). To explore the effects of TGF-β family of growth factors on these Nkx2.5^{+} cells, we treated FACS-purified eGFP^{+} cells from the neonatal heart with TGF-β1 (100 ng/ml), activin A (100 ng/ml) and BMP2 (100 ng/ml) for 7 days and examined the effects of these growth factors on cardiomyocyte and smooth muscle cell differentiation (Fig. 1A–C). As shown in Figure 1, treatment with TGF-β1 induced an eightfold increase in smooth muscle cell differentiation (from ~10% to 80% of total cells) and this effect can be abolished by co-treatment with A83 (1 μM), a small molecule inhibitor of Alk5. The other TGF-β family of cytokines, BMP2 and activin A, did not significantly increase smMHC[+] cell number (Fig. 1C).

The ability of TGF-β1 to induce smooth muscle cell differentiation of Nkx2.5[+] cardiomyoblasts is intriguing given that it is one of the three closely related TGF-β isoforms (TGF-β1, -β2 and -β3) that have been shown to act as cellular switches regulating immune function, cell proliferation and epithelial to mesenchymal transition [5, 10, 11, 14, 15]. Transforming growth factor-β1 first binds to specific type II serine/threonine kinase receptors termed TGF-βRII and then phosphorylates and activates a second serine/threonine kinase receptor, TGF-βRI [also called activin receptor like kinase (Alk) 5], or alternatively, Alk1 [16]. This complex then recruits and phosphorylates R-Smads (such as Smad2/3) to regulate transcription [13]. The ability of the SMAD signalling complex to regulate gene expression is dependent on its recruitment of co-activators (such as p300 and CBP) or co-repressors (such as TGIF, SKI and SnoN) [9]. The treatment of endothelial progenitor cells [17] and multi-potential 10T1/2 cells [18] with TGF-β1 have been shown to increase smooth muscle gene expression. Furthermore, the overexpression of either histone acetyltransferases (HAT) proteins (such as p300 and CBP) [19] or histone 3 lysine 9 (H3K9)-specific demethylase (Jmjd1a) [18] can enhance TGF-β1-induced SM22 promoter activities.

The selective ability of TGF-β1 but not activin A or BMP2 to induce smooth muscle cell differentiation of neonatal Nkx2.5[+] cardiomyoblasts is consistent with the known role of TGF-β1 on smooth muscle cell proliferation in aortic and pulmonary arteries. It is possible that Nkx2.5^{+} cells participate in the formation of coronary smooth muscle cells during development and this process is enhanced by an increase in the level of localized TGF-β1. Further studies will be needed to examine whether the treatment of neonatal hearts with TGF-β1 can result in increased coronary vessel development *in vivo*.

Small molecule inhibition of Alk receptors induces an expansion of neonatal Nkx2.5-eGFP^{+} cells

Our studies also uncovered a surprising aspect of TGF-β receptor regulation on the proliferation of neonatal Nkx2.5^{+} cells. We found
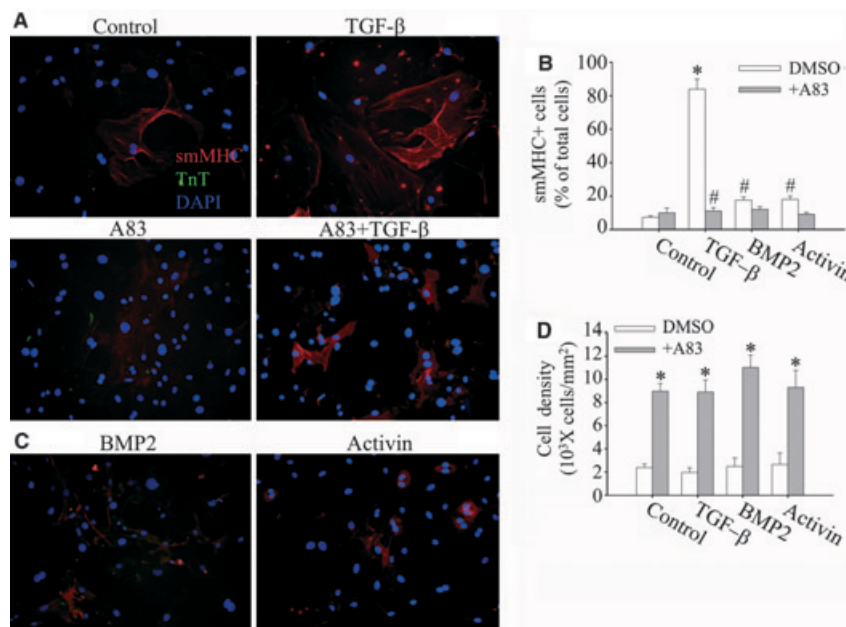

Fig. 1 Transforming growth factor (TGF)-β signalling regulates the proliferation and differentiation of postnatal Nkx2.5-GFP^{+} cells. Cells were fixed and immunostained by antibodies against troponin T (TnT-(fluorescein isothiocyanate (FITC), green) and smooth muscle myosin heavy chain (smMHC-Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC), red), and the nuclei were stained by DAPI (blue). (**A**) Effect of TGF-β1 and the TGF-βRI inhibitor, A83, on FACS-purified eGFP^{+} cells from the neonatal heart. (**B**) TGF-β1 (100 ng/ml), but not bone morphogenetic protein (BMP)2 and activin A, induced a greater number of smMHC^{+} cells following 7 days in culture. This effect could be abrogated by treatment with A83. (**C**) Effect of BMP2 (100 ng/ml) and activin (100 ng/ml) treatment on the differentiation of Nkx2.5^{+} cells. (**D**) Cell proliferation was enhanced after exposure to A83 (1 μM). Results represent the means from four independent experiments. *$P < 0.05$ *versus* DMSO in control group, and #$P < 0.05$ *versus* TGF-β + DMSO (by two-way ANOVA with Bonferroni–Dunn's *post-hoc* test).
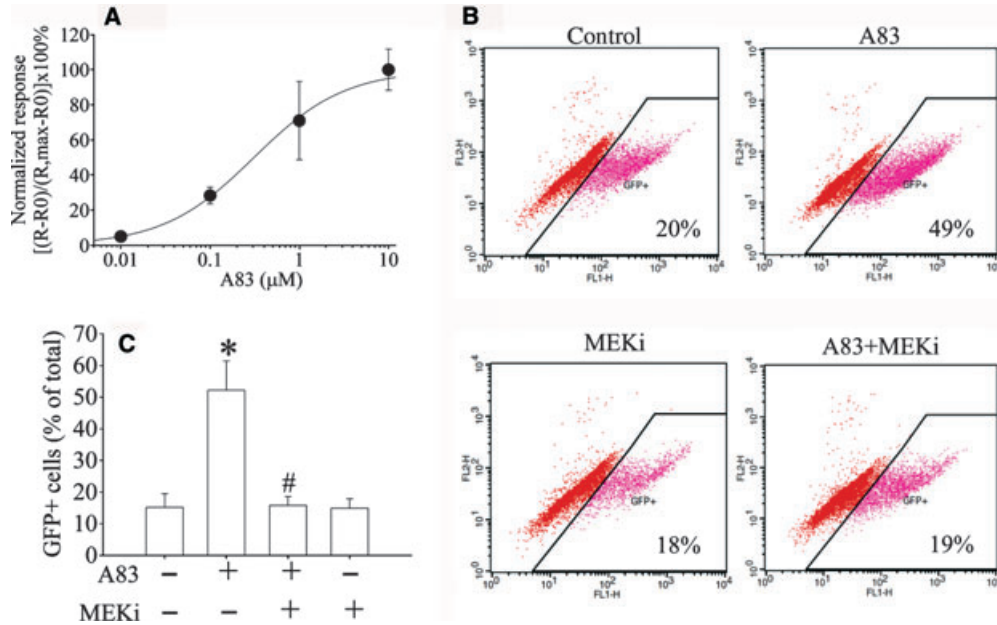
Fig. 2 A83 treatment increased Nkx2.5-GFP$^+$ cell proliferation and maintained eGFP expression. (**A**) Dose–response curve of A83 treatment on total cell number. The curve is fitted by Hill equation. EC$_{50}$ is 0.31 ± 0.05 μM and the Hill coefficient is 0.85 ± 0.10. Results represent mean ± S.E.M. of four independent experiments. (**B**) Flow cytometric analysis of the percentage of eGFP$^+$ cells in the absence (left panel) or presence (right panel) of A83. (**C**) Effect of transforming growth factor (TGF)-β and MEK inhibition on the percentage of eGFP$^+$ cells. Representative flow cytometry plot of FACS-purified eGFP$^+$ cells following treatment for 1 week with a MEK inhibitor (PD0325901) in the absence (left panel) or presence (right panel) of an Alk4, 5, 7 inhibitor (A83; right panel). Results are averaged from four independent experiments. *$P < 0.05$ control *versus* drug-treated group, and $^\#P < 0.05$ A83 *versus* A83 + MEKi (by one-way ANOVA with Dunnett's *post-hoc* test).

that inhibition of Alk5 with A83 markedly increased the number of Nkx2.5$^+$ cells and their progenies (~4.5-fold; Fig. 1D). This increase appears to be independent of signalling by TGF-β1, activin A or BMP2. The ability of A83 alone to induce the proliferation of Nkx2.5-GFP$^+$ cells is dose dependent with an EC$_{50}$ of 0.31 ± 0.05 μM and Hill's coefficient of 0.85±0.10 ($n = 4$; Fig. 2A).

To confirm that the cultured cells treated with A83 retained their expression of Nkx2.5 (*i.e.* eGFP), we performed flow cytometry analysis on FACS-purified eGFP$^+$ cells following treatment with A83 for 7 days and compared this with cells that have been treated with only Dimethyl sulfoxide (DMSO) as control. As shown in Figure 2B, the percentage of eGFP$^+$ cells decreased to 20.0 ± 4.3% of the total cells (from 100% at the beginning of the study) after 7 days of culturing in control condition and to 9.2 ± 4.1% in the presence of TGF-β1 (100 ng/ml; data not shown). On the other hand, treatment with A83 (1 μM) increased the percentage of eGFP$^+$ cells to 49.0 ± 9.3% ($P < 0.05$).

The maintenance of Nkx2.5-eGFP expression by A83 suggests that A83 may be either preventing smooth muscle cell differentiation of Nkx2.5$^+$ cells by blocking TGF-β signalling or that it has additional effects on cell proliferation. We examined whether the maintenance of eGFP$^+$ cell population by A83 can be modulated by inhibition of components of the Fibroblast Growth Factors (FGF) signalling pathway that is known to regulate cell proliferation and found that the A83-mediated proliferation effects could be abolished by concurrent treatment with a MEK inhibitor, PD0325901 (0.5 μM; Fig. 2C). While the exact influence that PD0325901 treatment has on the expansion of eGFP$^+$ cells remains to be worked out, our data suggest that a direct cross-talk between TGF-β and FGF signalling is important to maintain the Nkx2.5$^+$ cardiomyoblast phenotype.

In conclusion, we show here that TGF-β signalling plays an important role in regulating the proliferation and smooth muscle cell differentiation of neonatal Nkx2.5$^+$ CPCs. When employed in the appropriate clinical context, these small molecular inhibitors may be useful as therapeutic agents to enhance coronary vasculogensis and postnatal cardiac progenitor cell expansion.

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest related to this article.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Supplemental Materials and methods.

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