Original Article

MOLECULAR METABOLISM

A high-content small molecule screen identifies novel inducers of definitive endoderm

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ABSTRACT

Objectives: Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) can generate any given cell type in the human body. One challenge for cell-replacement therapy is the efficient differentiation and expansion of large quantities of progenitor cells from pluripotent stem cells produced under good manufacturing practice (GMP). FOXA2 and SOX17 double positive definitive endoderm (DE) progenitor cells can give rise to all endoderm-derived cell types in the thymus, thyroid, lung, pancreas, liver, and gastrointestinal tract. FOXA2 is a pioneer transcription factor in DE differentiation that is also expressed and functionally required during pancreas development and islet cell homeostasis. Current differentiation protocols can successfully generate endoderm; however, generation of mature glucose-sensitive and insulin-secreting β-cells is still a challenge. As a result, it is of utmost importance to screen for small molecules that can improve DE and islet cell differentiation for cell-replacement therapy for diabetic patients.

Methods: The aim of this study was to identify and validate small molecules that can induce DE differentiation and further enhance pancreatic progenitor differentiation. Therefore, we developed a large scale, high-content screen for testing a chemical library of 23,406 small molecules to identify compounds that induce FoxA2 in mouse embryonic stem cells (mESCs).

Results: Based on our high-content screen algorithm, we selected 84 compounds that directed differentiation of mESCs towards the FoxA2 lineage. Strikingly, we identified ROCK inhibition (ROCKi) as a novel mechanism of endoderm induction in mESCs and hESCs. DE induced by the ROCK inhibitor Fasudil efficiently gives rise to PDX1$^+$ pancreatic progenitors from hESCs.

Conclusion: Taken together, DE induction by ROCKi can simplify and improve current endoderm and pancreatic differentiation protocols towards a GMP-grade cell product for β-cell replacement.

Keywords Rock inhibition; Fasudil; Pancreatic progenitors; Anterior definitive endoderm; Differentiations

1. INTRODUCTION

The self-renewal capacity and the wide differentiation potential of pluripotent ESC lines have great therapeutic potential to treat chronic diseases such as diabetes mellitus, blindness, or neurodegeneration. Islet transplantation can cure brittle type 1 diabetic patients; however, donor islets are rare and preclude therapy for many patients. ESCs can represent an attractive source to produce functional β-cells from stem cells in the culture dish. Current problems include the expansion of large quantities of progenitor cells using GMP facilities and the generation of functional mature β-cells for transplantation [1]. The existing differentiation protocols are directly translated from developmental studies *in vivo* [2,3] and act by mimicking the developmental programs *in vitro* to facilitate generation and upscaling of pancreatic β-cells [4,5]. A major drawback of these protocols is the use of recombinant proteins and ligands that show variable activity and stability and are often exposed to animal products that might be contaminated with yet unidentified pathogens [4,5]. One strategy to overcome this problem and implement cheap and efficient GMP-grade ESC differentiation protocols is to replace biologics by small molecule compounds with stable and reproducible activity.

During embryogenesis, different developmental pathways regulate definitive endoderm (DE) formation and patterning, including the Wnt, fibroblast growth factor (FGF), transforming growth factor β (TGF- β)/Nodal/ActivinA (AA), bone morphogenic protein (BMP), and AKT/PI3K [6–9]. Modulating the signaling transduction events and genes involved in these pathways can help recapitulate the developmental processes *in vitro*. In current protocols, efficient DE induction is achieved by direct exposure of ESCs and iPSCs to recombinant proteins, including Wnt3a and AA [8]. However, these proteins can exhibit highly variable activity in culture, due to incomplete post-translational modifications, lack of stability of biological activity as well as batch-to-

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batch variations. As it is well known that Wnt and Nodal/TGF-β signaling gradients induce and pattern the mesoderm and endoderm germ layer [10], this can result in variable efficiencies of DE patterning *in vitro* from one week to another. Induction of heterogeneous DE populations can lead to a great inconsistency in establishing long-term differentiation protocols over 20–40 days towards one particular cell fate [4,5].

Small molecules can serve as tools to replace current proteins and induce the differentiation of ESCs. These molecules can effectively act on target proteins thereby modulating different signaling pathways [11]. The major advantage of using small molecules is that they can be synthesized in high amounts and with higher purity and stored in a way that the substances have reproducible activity. High-throughput screens to monitor directed endodermal differentiation have been reported previously [11,12]. These screens introduce small molecules that modulate the TGF-β pathway, replacing the use of AA in differentiation cocktails to induce endoderm; however, there is still a great need to identify novel potent endoderm inducers that can effectively augment terminal pancreatic differentiation protocols [4,5,11,13,14]. Towards this aim, we set-up a high-content screen in mESCs and tested 23,406 small molecules. We identified the Rho associated coiled like protein kinase (ROCK) inhibitor Fasudil as a small molecule that efficiently induces DE in both mESCs and hESCs. Moreover, when compared with the traditional Wnt3a and AA endoderm induction cocktail, ROCKi treated cells showed similar differentiation towards DE. We show that another analogue of Fasudil, RKI-1441, showed similar differentiation efficiencies of mESCs and hESCs towards DE indicating that ROCKi is sufficient to induce DE in culture. Furthermore, the ROCKi differentiates the PSCs towards anterior definitive endoderm (ADE), which gives rise to thymus, thyroid, lung, liver, and pancreas. We found that ROCKi does not induce extraembryonic visceral endoderm or mesoderm in the cell culture system. Additionally, ROCKi-induced DE from hESCs differentiated efficiently into pancreatic progenitors (PP), suggesting a supportive role of ROCKi in pancreatic differentiation. Altogether, we introduce a family of small molecule ROCKis and a novel mechanism that can robustly induce DE/ADE differentiation of PSCs in culture thereby replacing biologics in the differentiation medium.

2. METHODS AND MATERIALS

2.1. Culture, maintenance, and differentiation of mouse and human embryonic stem cells

In-house made (IDG) mESCs (FoxA2-Venus/Oct3/4-RFP) were thawed on mitomycin treated feeders and maintained undifferentiated in ES medium based on DMEM (41966-052; Gibco) containing 15% FCS (PAA, A15-108), mLIF (self-made), 12 ml HEPES (2503024, Gibco), 5 ml Penicillin/Streptomycin (15140122; Gibco), and 1 ml 2-mercaptoethanol (Gibco, 31350-010). *In vitro* differentiation of the mESCs towards endoderm was carried out in monolayer on 0.1% gelatine coated dishes. The cells were mouse embryo fibroblast feeder cells (MEF) depleted and cultured for few consecutive passages on gelatine and ES medium. On the day of differentiation, mESCs were seeded on gelatine coated dishes directly in serum free medium (SFM). SFM was based on advanced DMEM/F-12 (12634028, Gibco) and advanced RPMI (12633020, Gibco) in ratio 1:1.500 ml of SFM was supplemented with 10 ml GlutaMax™ (35050061, Gibco), 12 ml HEPES (2503024, Gibco), 5 ml Penicillin/Streptomycin (15140122, Gibco), Cytidine 20 ng/ml final concentration (C122106, Sigma Aldrich). For control endoderm differentiation, SFM was supplemented with 1 ng/ml of murine Wnt3a (1324 WN-CF, R and D systems) and 12.5 ng/ml of Activin A (338-AC, R and D systems). Freshly prepared SFM supplemented with Wnt3a and Activin A was added every day until day 4 to achieve optimum differentiation of mESCs towards definitive endoderm. Compound induced differentiation was achieved by adding 3 μM of Fasudil or other ROCK inhibitors to the SFM instead of Wnt3a and Activin A. The differentiation efficiency was assessed based on endogenous expression of FoxA2 and Sox17.

For human ESCs (H9), the cells were thawed on 1:30 geltrex coated dishes along with 10 μM of Y compound for 1 day. The cells were maintained in iPS-Brew medium (130-104-368; Miltenyi Biotech). For differentiation, single cells were seeded on geltrex coated 6-well plates (2 million cells/well). On day 1, the cells were treated with 20 ng/ml WNT3A and 100 ng/ml AA or 10 μM of the ROCK inhibitors for 4 days for DE induction.

2.2. Screening setup for unknown novel compounds that can induced endoderm differentiation in mESCs

2.2.1. Assay seeding and medium change with re-addition of fresh compound solution

For screening, 384 well Cell Carrier plates (Perkin Elmer) were coated with 0.1% gelatine solution. After discarding coating solution, compounds were spotted by echo liquid handling system (Labcyte Echo 550). For primary screening, 250 nl of 20 mM compound solution in 100% DMSO was spotted to the compound area. Coated assay plates with fresh compound solution were stored at 16 °C over night. 1–2 h before seeding the ESCs onto assay plates, ESC medium was changed. Directly before seeding, the cells were washed with PBS (without $Ca2^{+}$ and $Mg2^{+}$) and trypsinized. Cells were resuspended in SFM, and cell density was adjusted to 220.000–280.000 cells/ml (optimized for each batch). For the compound area, 50 μl of the cell suspension was added using Multidrop (Thermo Labsystems Type 832). The following controls were used for the assay: a) Differentiation control: Activin A and Wnt3a (final concentrations of 12.5 ng/ml (Activin A) and 1 ng/ml (Wnt3a) plus 0.5% DMSO, b) pluripotency control: GSK3β Inhibitor (CHIR 99021) at 3 μM final concentration in 0.5% DMSO, c) negative controls: 0.5% DMSO).

In the primary screen and hit confirmation single dose, compounds were tested at a final concentration of 10 μM. On day 2 post seeding, the medium was changed (fresh SFM + compound or control substances). For the medium change procedure a pre dilution plate (with compounds) was prepared in advance: Here, compounds (300 nl/well) were spotted into sterile round bottom PP 384 well plates (Greiner, 784201). Then, 60 μl SFM was added using Multidrop to give final compound concentration of 10 μM at 0.5% DMSO. For control wells SFM was supplemented with growth factors, GSK3β Inhibitor or DMSO, 60 μl of control medium cocktail was added to the appropriate wells of the pre-dilution plate (according to the plate layout): a) differentiation control: 12.5 ng/ml Activin A + 1 ng/ml Wnt3a, b) pluripotency control: 3 μM GSK3β Inhibitor (CHIR 99021) and, c) 0.5% DMSO solvent control (Supplementary Figures 3 and 4). For complete medium change, the medium of the assay plate was discharged and fresh medium from the pre-dilution plate was transferred to the assay plate using Perkin Elmer Janus Mini AJ SM001/384 Tip MDT with sterile Tips (slowest dispensing speed, without touching the cells). After medium change assay, plates were immediately placed into an incubator (37 °C and 5% CO2) until day 5 after seeding.

2.3. Assay plate processing and immunostaining

On day 5 post seeding, assay plates were washed 1× with PBS and fixed for 5 min at RT with 4% PFA, rinsed 1× in PBS and 2× in PBST

