Induction of Pax3 gene expression impedes cardiac differentiation

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Cell-based therapies using pluripotent stem cells hold great promise as regenerative approaches to treat many types of diseases. Nevertheless many challenges remain and, perhaps foremost, is the issue of how to direct and enhance the specification and differentiation of a desired cell type for potential therapeutics. We have examined the molecular basis for the inverse correlation of cardiac and skeletal myogenesis in small molecule-enhanced stem cell differentiation. Our study shows that activation of premyogenic factor Pax3 coincides with inhibiting gene expression of early cardiac factor GATA4. Interestingly, the inhibitory effect of small molecules on cardiac differentiation depends on the function of Pax3, but not the mesoderm factor Meox1. Thus Pax3 is an inhibitor of cardiac differentiation in lineage specification. Our studies reveal the dual roles of Pax3 in stem cell fate determinations and provide new molecular insights into small molecule-enhanced lineage specification.
Results

Bexarotene inhibits the commitment of ES cells into cardiomyocytes. Since the early events of embryonic myogenesis is recapitulated closely by differentiation of ES cells into cardiac and skeletal muscle lineages24,25, we used an embryoid body (EB) formation approach to study the impact of small molecules on myogenic conversion of ES cells. The ES cells were cultivated in the hanging drops for 2 days to form the EBs which were then cultured in suspension for 5 days and maintained as adhering culture for additional 10 days (Fig. 1A). For immunofluorescence microscopy analysis, the cells were co-stained for myosin heavy chain and MyoD to quantify the development of cardiac and skeletal myocytes. Developed cardiac and skeletal myocytes are distinguished by their staining pattern and morphological characteristics. Skeletal myocytes were recognized as elongated bipolar myocytes positively stained for myosin heavy chain and MyoD, while cardiac myocytes are rounded myosin heavy chain positive but MyoD negative cells.

Cultured in media favorably for differentiation of skeletal myocytes but in the absence of small molecule inducers, ES cells were able to spontaneously differentiate into cardiac and skeletal myocytes but at low frequencies. Only about 1.5% of cardiac and 1% of skeletal myocytes were generated as determined by the quantitative immunofluorescence microscopy (Fig. 1B and C). In line with the literature2, treatment of the ES cells with RA during day 2–5 of EB formation, attenuated the generation of cardiac myocytes while enhancing skeletal myogenesis moderately to about 3% (Fig. 1B and C).

Bexarotene, a selective ligand of retinoid X receptor (RXR), is a more efficient inducer for ES cells to differentiate into skeletal myocytes than RA26. To delineate the mechanisms of bexarotene action, we examined the impact of bexarotene on the specification of cardiac lineage in ES cell differentiation. As shown in figure 1B and C, bexarotene inhibited the differentiation of ES cells into cardiac myocytes, while markedly increased the specification of skeletal muscle lineage to about 11%. Moreover, the quantitative real-time RT-PCR analyses revealed that bexarotene increased the transcripts of Meox1 more potently than RA in the EBs, whereas it was less efficient at increasing the transcripts of Pax3 (Fig. 1D). Thus, our findings establish that bexarotene, is also an inhibitor of cardiac differentiation, similar to RA in ES system, if administered during EB formation.

Inhibition of GATA4 early expression by bexarotene. Pluripotent P19 stem cells can be directed into differentiation to form cell lineages of all three germ layers27,28. They have served as a valuable model system to study myogenesis and to identify small molecule inducers for lineage specification27,28. Specifically, the differentiation of P19 cells into cardiac and skeletal myocytes reflects the cellular and molecular processes occurring in early embryogenesis and ES cell differentiation29. In tissue cultures, the P19 cells can be induced into differentiation with an aggregation protocol that involves the conversion of ES cells. The ES cells were cultivated in the hanging drops for 5 days and maintained as adhering culture for additional 10 days (Fig. 1A). For immunofluorescence microscopy analysis, the cells were co-stained for myosin heavy chain and MyoD to quantify the development of cardiac and skeletal myocytes. Developed cardiac and skeletal myocytes are distinguished by their staining pattern and morphological characteristics. Skeletal myocytes were recognized as elongated bipolar myocytes positively stained for myosin heavy chain and MyoD, while cardiac myocytes are rounded myosin heavy chain positive but MyoD negative cells.

As shown in figure 2B and C, DMSO induced about 8% of cardiac and 6% of skeletal myocytes in the P19 cells, respectively, as quantified by quantitative immunofluorescence microscopy, consistent with previous studies. Cotreatment of the EBs with RA enhanced the generation of skeletal myocytes, while attenuating cardiac differentiation (Fig. 2B and C), as previously reported. Bexarotene enhances the specification of muscle lineage in a concentration dependent manner and the efficacies of bexarotene on the differentiation of P19 cells into skeletal myocytes is comparable to RA26. More interestingly, bexarotene also inhibited the generation of cardiac myocytes by P19 cells in a concentration dependent manner (Fig. 2B and C). While higher concentrations of bexarotene enhanced the specification of skeletal muscle lineage to about 20%, it markedly impeded cardiac differentiation (Fig. 2B and C). Nevertheless, this negative effect of bexarotene on P19 cardiac differentiation appears to be less potent than RA (Fig. 2C).

Western analysis revealed that the expression of GATA4, an early cardiac factor, was induced by day 4 of differentiation following treatment of the EBs with DMSO (Fig. 2D). This early expression of GATA4 gene was notably impaired by the administration of bexarotene or RA (Fig. 2D). Again, RA appears to be more effective than bexarotene on the inhibition of GATA4 gene expression. However, the expression of myogenin protein, a specific skeletal muscle factor that was enhanced by bexarotene or RA to a similar degree on day 9 of differentiation, correlating with their efficacies on the specification of skeletal muscle lineage (Fig. 2B–D). The expression of Meox1 and Pax3 was increased by bexarotene and RA by day 4 of differentiation. Similar to ES cell differentiation, bexarotene increased the level of Meox1 gene expression more than RA, whereas RA had a higher impact than bexarotene on the expression of Pax3 gene, by about 2-fold (Fig. 2E and F). Thus, the temporal gene expression pattern induced by bexarotene during P19 myogenic differentiation is similar to skeletal myogenesis in vivo and in ES cell differentiation. In addition, the inhibitory effects of bexarotene and RA on cardiac biology becomes more potent.
because Meox1 is important for skeletal myogenesis. Regardless, the dominant negative Meox1 cells into skeletal myocytes, they were still able to generate cardiac myocytes, albeit with a lesser degree in comparison to DMSO treated cells (Fig. 3A). Thus, the function of Pax3 is important for bexarotene- and RA-inhibited cardiac differentiation.

Interestingly, while the transcript levels of EnR remained constant following different treatments (Fig. 3B), the negative effect of bexarotene on cardiac differentiation was less potent than RA just as in the wild-type cells (Fig. 3A). Similar to the wild-type cells, bexarotene had a larger effect than RA on Meox1 gene expression, about 2-fold, in the Pax3/EnR cells, whereas RA was more effective at inducing My5 gene expression (Fig. 3C–D). In addition, DMSO effectively induced the gene expression of cardiac factors, GATA4, Nkx2.5 and Tbx5 in the Pax3/EnR cells (Fig. 3E–G). Importantly, the inhibitory effect of bexarotene on the transcript levels of GATA4, Nkx2.5 and Tbx5 was less potent than RA, correlating with their efficacy on the inhibition of cardiac differentiation (Fig. 3A and E–G). Taken together, our data suggest that bexarotene and RA inhibits cardiac differentiation in part through the activation of Pax3 gene expression which appears to play dual roles in stem cell fate determinations.

**Discussion**

We have examined the molecular basis on the inverse correlation of cardiac and skeletal myogenesis in small molecule-enhanced stem cell differentiation. Small molecule inducers such as RA and bexarotene are able to enhance the generation of skeletal myocytes by activating premyogenic transcription factors at the stage of lineage specification. Our findings show that the activation of premyogenic factor Pax3 coincides with inhibiting gene expression of early cardiac factors such as GATA4, Tbx5 and Nkx2.5. Interestingly, the inhibitory effect of bexarotene and RA on cardiac differentiation requires the function of Pax3, but not Meox1. Thus Pax3 is an inhibitor of cardiac differentiation in lineage specification. Our studies reveal the dual roles of Pax3 in stem cell fate determinations and provide new molecular insights into small molecule-enhanced lineage specification.

Pluripotent stem cells, regardless of their origin, possess the potential of developing into many types of cell lineages. The central issue is
how differentiation cues commit the cells into a particular lineage and what impact this would have on the specification of other cell lineages. Understanding the molecular mechanisms of lineage specification is central to devise the best strategies for preferentially enhancing lineage specific differentiation in an efficacy suitable for potential therapeutics. Consistent with the literature, RA is able to enhance skeletal myogenesis in pluripotent stem cells, but inhibits cardiac differentiation if used at the stage of lineage specification (Fig. 1 and 2). We also found that bexarotene has similar effects as RA on cardiac differentiation (Fig. 1 and 2). This is particularly interesting as we have previously established that bexarotene activates the skeletal muscle program through a mechanism distinct from RA-mediated pathway.

In addition, our data also revealed a role of Pax3 in cardiac differentiation. Pax3 is important for skeletal myogenesis, but overexpression of Pax3 leads to a loss of cardiac differentiation. Our studies establish Pax3 as a molecular pathway of small molecule-enhanced skeletal myogenesis, but -inhibited cardiac differentiation (Fig. 3). Although the specification events differ between skeletal and cardiac lineages, they both are from mesoderm origin. During in vitro stem cell differentiation, lineage specification factors apparently not just exert their functions through the activation of the lineage they command, but also through the inhibition of other lineages to compete for the pool of progenitor cells, when spatial partition is nonexistent. While skeletal and cardiac myogenesis may be segregated spatially during embryogenesis, small molecules are effective means to employ the dual roles of lineage specific factors for generating specific lineage from pluripotent stem cells for potential therapeutics.

Our studies have not examined the genomic targets of Pax3 in relation to cardiac inhibition. Systems studies are required to uncover additional targets of Pax3 involved in the inhibition of cardiac differentiation. Understanding the molecular mechanisms of myogenic conversion is central to direct and enhance lineage specification in cell-based therapies. We have identified a potential mechanism for lineage competition, which suggests early lineage factors regulate lineage commitment by regulating and integrating different signaling pathways. The dual roles of lineage specific factors in stem cell fate determination provide interesting avenue in identifying effective molecular targets to convert pluripotent stem cells into desired cell lineage.

**Methods**

**Cell culture and differentiation.** ES Cells (ATCC) were maintained in Dulbecco’s modified Eagle medium (D-MEM) (Gibco-Invitrogen) supplemented with 15% fetal bovine serum (PAA), 1% non essential amino acids (Gibco-Invitrogen) and 1.18 mM β-mercaptoethanol (OmniPur) at 37°C with 5% of CO₂. Maintenance cultures were supplemented with 1,000 units/ml of Leukemia Inhibitory Factor (Chemicon). For differentiation, ES cells were cultivated in hanging drops on inverted lids for 2 days and in Petri dishes for additional 5 days to form EBs which were then transferred into tissue culture dishes or onto coverslips coated with 0.1% gelatin and grown for another 10 days. P19 cells (ATCC) were maintained in minimum essential medium α (α-MEM) (Gibco-Invitrogen) supplemented with 5% fetal bovine serum (PAA) and 5% bovine calf serum (PAA) at 37°C with 5% of CO₂. For differentiation, cells were aggregated in Petrie dishes for 4 days to form EBs. The EBs was then transferred into tissue culture dishes or onto coverslips coated with 0.1% gelatin and grown for a further 5 days. All-trans retinoic acid (RA) was purchased from the Sigma-Aldrich, and bexarotene was from the LC Laboratories.

**Immunofluorescence microscopy.** Following differentiation, cells were fixed on the coverslips with cold methanol, rehydrated with PBS, and incubated with the appropriate primary antibodies overnight at 4°C, and then with the corresponding fluorescent secondary antibodies at room temperature for 2 hours. After washing with PBS, the cells were incubated with Hoechst (Molecular Probes) for 3 minutes to stain the DNA. Finally, the coverslips were mounted on slides with 50% glycerol. Microscopy analysis was performed with the Zeiss Axiosvert 200 M microscope. Cells were observed through a Zeiss AxioCam HRm monochrome camera. Myogenesis was assessed as the percentage of cells stained positively for skeletal or cardiac markers in relation to total cell populations. Images captured through different fluorescence filters were processed and merged by the Zeiss AxioVision Rel 4.6 software. The primary antibodies used were myosin heavy chain (MF20) and MyoD (Santa Cruz).
Real-Time RT-PCR. Total RNA was isolated using RNeasy Mini kit (Qiagen) and reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was conducted by using the Applied Biosystems 7500 Fast Real-Time PCR System. The amount of targets, normalized to the GAPDH endogenous reference and relative to calibrator control is calculated using the arithmetic formula 2^(-ΔΔCT). The primers used for the following: Meox1 fwd- GAGAGGCAGCAACAGCCAAGGAG Meox1 rev- CGTAGCTGCTCCTGTTGGAAG Pax3 fwd- GTCGACTGACAGACTGGGAAAC Pax3 rev- GAAGAGACTTGGCCTAATCAGTC EnK fwd- TCGTGGTTGACGACGTTG EnK rev- GCTGCTGCTGGAGA A Myf5 fwd- GCCATGTCGTAAGTTAACAGC Myf5 rev- CAAATCCAAGCTGCACAGGAA GATA4 fwd- GAAGACACCCCAATCTCGATATG; GATA4 rev- GGCATTGCACAGGTGTCC GATA4 fwd- GAGAACCCCACTTCTGATATG; GATA4 rev- GCCATTGTACAGTGTC GATA4 rev- GCCATTGTACAGTGTC Nkx2.5 fwd- AGTGCCTCCTGCGTCTTTCCA; Nkx2.5 rev- ATCCGGTCTGGTTTCTGC Tbx5 fwd- AACAGTACGAGCTTCTGG Tbx5 rev- GAGGGTATCTTCTGGCTGT C

Western analysis. Cells were lysed by incubation with whole cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% NP-40) for 30 minutes on ice and centrifuged at 12,000 g for 15 minutes to collect the supernatant. Protein concentrations were assessed by Bradford Method. Equal amounts of protein were separated on SDS-PAGE and transferred onto Immob-Blot PVDF membrane (Bio-Rad). Proteins were then visualized using Western Lightning Chemiluminescence reagents (Perkin Elmer). The primary antibodies used were GATA4 (Santa Cruz), myogenin (FSD) and β-tubulin (E7).

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Author contributions
Q.L. and J.C. designed the research, interpreted the data and prepared the manuscript. M.L. and F.L. performed the experiments. M.I., L.C., D.R., L.C., and K.K. analyzed the data. Q.L. and J.C. designed the research, interpreted the data and prepared the manuscript. M.L. and F.L. performed the experiments. M.I., L.C., D.R., L.C., and K.K. analyzed the data.

Additional information
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