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Lineage specification of Flk-1$^+$ progenitors is associated with divergent Sox7 expression in cardiopoiesis

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ABSTRACT

Embryonic stem cell differentiation recapitulates the diverse phenotypes of a developing embryo, traceable according to markers of lineage specification. At gastrulation, the vascular endothelial growth factor (VEGF) receptor, Flk-1 (KDR), identifies a mesoderm-restricted potential of embryonic stem cells. The multi-lineage propensity of Flk-1$^+$ progenitors mandates the mapping of fate-modifying co-factors in order to stratify differentiating cytotypes and predict lineage competency. Here, Flk-1-based selection of early embryonic stem cell progeny separated a population depleted of pluripotent (*Oct4*, *Sox2*) and endoderm (*Sox17*) markers. The gene expression profile of the Flk-1$^+$ population was notable for a significant upregulation in the vasculogenic *Sox7* transcription factor, which overlapped with the emergence of primordial cardiac transcription factors *GATA-4*, *Myocardin* and *Nkx2.5*. Sorting the parental Flk-1$^+$ pool with the chemokine receptor CXCR4 to enrich the cardiopoietic subpopulation uncovered divergent *Sox7* expression, with a 7-fold induction in non-cardiac compared to cardiac progenitors. Bioinformatic resolution sequestered a framework of gene expression relationship between Sox transcription factor family members and the Flk-1/CXCR4 axes with significant integration of β-catenin signaling. Thus, differential *Sox7* gene expression presents a novel biomarker profile, and possible regulatory switch, to distinguish cardiovascular pedigrees within Flk-1$^+$ multi-lineage progenitors.

1. Introduction

Embryonic stem cells harbor a consistent capacity for multi-lineage differentiation (Dimmeler et al., 2005; Murry et al., 2005, van Laake et al., 2005; Srivastava and Ivey, 2006). With a unique ability to balance the processes of proliferation and lineage specification, embryonic stem cells provide a self-renewing pool of progenitors capable of differentiation into distinctive functional and structural cardiac phenotypes (Behfar et al., 2002; Sachinidis et al., 2003; Menasche, 2005; van Laake et al., 2006). Stem cell-based cardiopoiesis originates from undifferentiated progenitors that are specified into early cardiac mesoderm (Foley and Mercola, 2004; Olson, 2006; Martin-Puig et al., 2008). Then, transition into cardiac lineage-restricted differentiation occurs with nuclear translocation of cardiac transcription factors, culminating with metabolic maturation and establishment of efficient ionic handling required for excitation–contraction coupling (Behfar et al., 2007; Chung et al., 2007; Perez-Terzic et al., 2007). The cardiogenic capacity of embryonic stem cells has been associated with therapeutic benefit that include re-muscularization of infarcted myocardium and repair of non-ischemic cardiomyopathic hearts, leading to improved contractile performance and reduced overall mortality (Hodgson et al., 2004; Menard et al., 2005; Kolossov et al., 2006; Nelson et al., 2006; Singla et al., 2006; Yamada et al., 2008). Yet, the risk of unregulated growth and neoplastic transformation, inherent to pluripotency, limits therapeutic applications (Behfar et al., 2005; Laflamme and Murry, 2005; Behfar and Terzic, 2007; Passier et al., 2008). Establishing a predictive molecular map to navigate pluripotent background would facilitate strategies that identify and select cardiac-restricted progenitors.

Within the developing embryo, tissue-specific programs guide progenitor cell commitment in highly organized temporal and spatial patterns, eliminating non-specific growth and sporadic differentiation (Latif et al., 2006; Wu et al., 2008). Vascular endothelial growth factor (VEGF) receptor-2 (Flk-1 or KDR) is associated with distinct mesoderm-restricted progenitors according to a biphasic expression profile during embryogenesis in which early expression marks hematopoietic lineages and delayed expression identifies cardiovascular progenitor cell potential (Nishikawa et al., 1998; Hidaka et al., 1999; Kattman et al., 2006; Yang et al., 2008). Separating a cardiogenic subpopulation from a pool of randomly differentiating embryonic stem cell progeny has been recently achieved based on dual cell surface expression of the Flk-1 multi-lineage mesoderm marker and the

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mesendoderm marker, fusin (CXCR4; Nelson et al., 2008). Upon cardiac specification of embryonic tissue, transcription factors drive a deterministic cascade of genetic programs to secure cardiogenesis (Buckingham et al., 2005; Faustino et al., 2008). Decoded networks of the pre-cardiac mesoderm unearthed candidate genes associated with the regulation of progenitor cell fate.

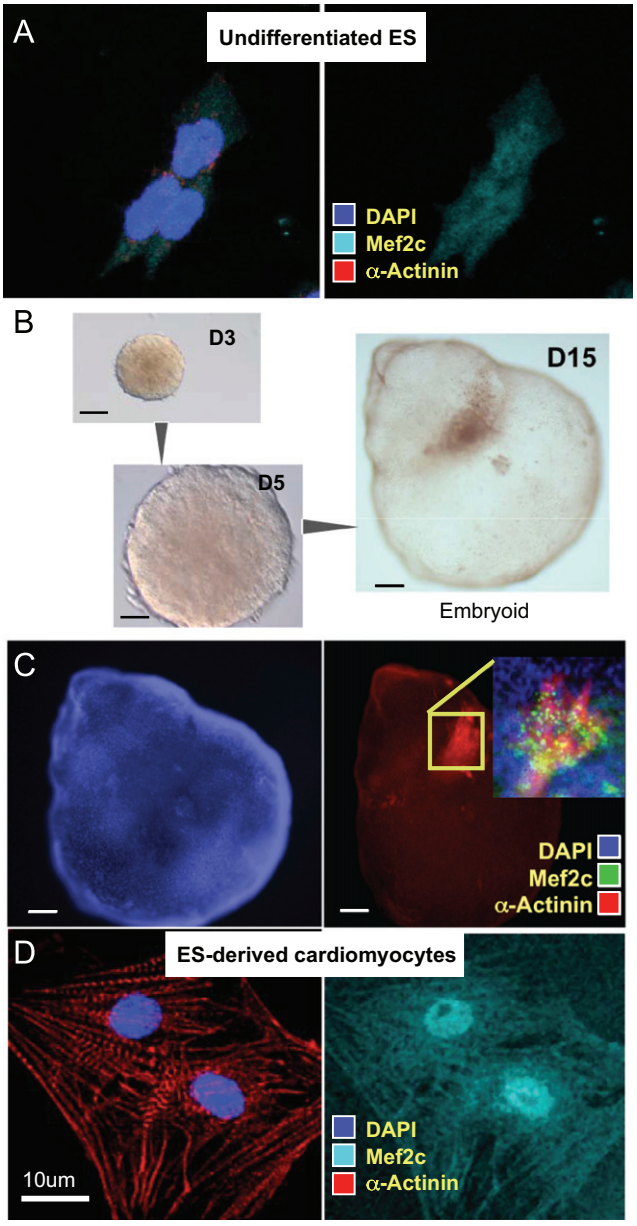

Fig. 1. Embryonic stem cell-derived cardiogenesis occurs during spontaneous differentiation. (A) Embryonic stem cells identified by nuclear staining (blue) do not express cardiac sarcomeric α-actinin (red staining, left panel) or the cardiac transcription factor Mef2c (cyan staining, right panel). (B) Embryonic stem cells are differentiated using the hanging drop method to form aggregates that develop into small clusters after 3 days of growth (D3) and continue to enlarge as a solid mass through day 5 (D5), giving rise to a cystic structure resembling the gross appearance of the developing embryonic tissues (D15). Bars, 10 μm. (C) Embryoids contained a band of tissues expressing the cardiac transcription factor, Mef2c, in a punctate nuclear pattern overlapping with cytoplasmic expression of sarcomeric α-actinin (insert, right panel). Non-cardiac tissues as identified by nuclear staining with DAPI surrounded the centrally located patch of Mef2c/α-actinin positive cardiac tissue ($n = 50$). Bars, 10 μm. (D) Isolated cardiomyocytes purified from embryonic stem cell-derived embryoids demonstrate sarcomeres expressing α-actinin (red staining, left panel), and nuclear localization of cardiac transcription factor Mef2c (cyan staining, right panel). Bar, 10 μm.

In this study, Flk-1 provided a tag to isolate embryonic stem cell-derived multi-lineage progenitors containing a pre-cardiac mesoderm subpopulation enriched by co-expression with CXCR4. Cardiac from non-cardiac fate was characterized by the divergent expression of *Sox7*, a member of the F-group of Sox family transcription factors, identifying a biomarker that stratified Flk-1$^+$ progeny in early stem cell differentiation.

2. Methods

2.1. Embryonic stem cell differentiation

The embryonic CGR8 stem cell line was maintained in Glasgow's Minimum Essential Medium (BioWhittaker-Cambrex, Walkersville, MD) supplemented with pyruvate (Cellgro, Mediatech, Inc. Herndon, VA), non-essential amino acids (Cellgro, Mediatech, Inc. Herndon, VA), β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 7.5% fetal calf serum (FCS, Invitrogen Corporation, Carlsbad, CA) and leukemia inhibitory factor (LIF; ESGRO, Chemicon International, Inc., Temecula, CA). Embryonic stem cells were placed in ~2500 standard hanging drops that initially contained 250 cells each, and were cultured for 2 days in 20% FCS (Invitrogen Corporation, Carlsbad, CA) supplemented with 30 ng/ml TNF-α (Invitrogen Corporation, Carlsbad, CA) in the absence of LIF and then allowed to further differentiate as large aggregates in suspension for 3 additional days (Perez-Terzic et al., 2003; Behfar et al., 2007). A time course of differentiation starting with embryonic stem cells maintained in LIF was obtained from cell aggregates in suspension at days 3–5, and from plated embryoid bodies at days 7–9. Dual interface Percoll gradient was used to separate the lower density sarcomere-poor cardiopoietic phenotype from the sarcomere-rich high-density cardiomyocytes (Behfar et al., 2007). Derived cells were fixed in 3% paraformaldehyde, permeabilized with 1% Triton X-100, and immunostained with antibodies specific for cardiac transcription factors Mef2c (1:400, Cell Signaling Technologies, Danvers, MA) and the sarcomeric protein α-actinin (1:1,000, Sigma-Aldrich, St Louis, MO) along with DAPI staining to visualize nuclei (Behfar et al., 2007; Nelson et al., 2008). Microscopy was preformed using an LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY).

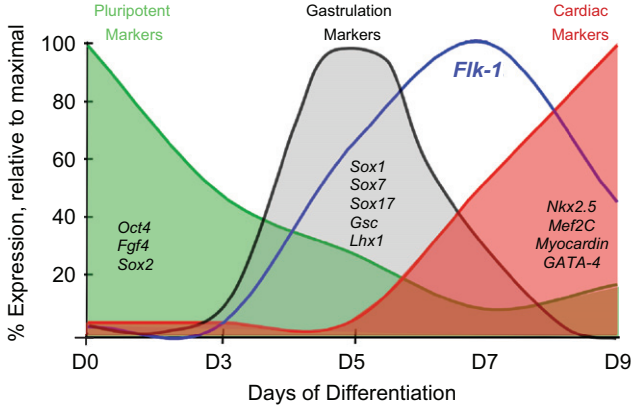

Fig. 2. Transcriptional profile kinetics of embryonic stem cell progeny preceding cardiogenic induction. Gene expression was determined by RT-PCR and graphed relative to maximal gene expression levels within the 9-day period of differentiation. The pluripotent transcriptome, including *Oct4* and *Fgf4* genes, demonstrated the highest expression levels at the earliest stages of differentiation. Expression of genes associated with gastrulating tissue, including *Sox1*, *Sox7*, *Sox17*, *Gsc* and *Lhx1*, peaked around day 5. Flk-1 gene expression bridged the transition between gastrulating tissue at day 5 and the onset of cardiac transcription factor expression at day 7. The cardiogenic transcriptional profile was characterized by initial expression of *Nkx2.5*, *Mef2C*, *Myocardin* and *GATA-4*.