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

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SUMMARY

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 gene expression relationships 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.

Keywords

bioinformatics; biomarker; cardiovascular; CXCR4; progenitor; selection; stem cells

INTRODUCTION

Embryonic stem cells harbor a consistent capacity for multi-lineage differentiation (Dimmeler et al, 2005; Murry et al, 2005, Van Kaake 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

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CONFLICT OF INTEREST STATEMENT

Authors disclose no conflicts of interest.

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and structural cardiac phenotypes (Behfar et al, 2002; Sachinidis et al, 2003; Menasche, 2005; Van Lakke 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; Ménard 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 pattern, eliminating non-specific growth and sporadic differentiation (Latif et al, 2006; Wu et al, 2008). Vascular endothelial growth factor 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 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 unearth candidate genes associated with the regulation of progenitor cell fate.

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.

METHODS

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), 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 ~2,500 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 day 3–5, and from plated embryoid bodies

at day 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 performed using an LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Cell flow sorting

Day 5 aggregates were washed in PBS, and then dissociated using non-enzymatic dissociation buffer (Invitrogen Corporation, Carlsbad, CA) for 10 min at 37°C (Nelson et al, 2008). Aggregates were triturated using 10 ml pipette until single cell suspension was obtained. Derived cells were spun down at 1,000g for 5 min and resuspended in propagation media (7.5% FCS) for 10 min to allow cells to recover, prior to collection and labeling for cell surface expression. Cells were washed with PBS and resuspended in 1 ml PBS which contained PE-conjugated primary antibody for Flk-1 (1:200, BD Biosciences, San Jose, CA) and then incubated on ice for 30 min, followed by single 10 ml PBS wash. In a subset of experiments, cells were co-incubated with goat-CXCR4 antibody (1:150, Abcam, Cambridge, MA) with secondary anti-goat Alexa 488 (1:500, Molecular Probes, Invitrogen Corporation, Carlsbad, CA). Cells were isolated using a FACS Vantage SE flow cytometer (BD Biosciences, San Jose, CA). PE was excited with the 488 nm laser line, and detected through a 575/26 bandpass filter. Alexa 488 was excited with a 488 nm argon laser, and detected through a 530/30nm bandpass filter. Forward and side scatter parameters were used to collect viable cell populations into standard cell culture medium. Sorted cell populations were centrifuged, and either frozen in liquid nitrogen for subsequent RNA isolation or plated on gelatinized cell culture plates for *in vitro* differentiation.

Gene expression profiling and bioinformatic network analysis

Total RNA was extracted from cell populations using a combination of gDNA Eliminator and RNeasy columns (Qiagen, Valencia, CA). cDNA was prepared from total RNA samples using MMLV Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA). Real-time PCR was performed using a standard TaqMan[®] PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The 50 μ l PCR included 3 μ l RT product, 25 μ l $\times$ TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 19.5 μ l RNase free water and 2.5 μ l TaqMan[®] Gene Expression Assays (pre-designed, pre-optimized probe and primer sets for each gene of interest, Applied Biosystems, Foster City, CA). TaqMan[®] Gene Expression Assays contained 2 unlabeled PCR primers (900 nM each final concentration) and 1 FAM dye-labeled TaqMan[®] MGB probe (250 nM final concentration). Reactions, run in triplicate, were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (C_T) was defined as the fractional cycle number at which fluorescence passes the fixed threshold. TaqMan[®] C_T values were converted into relative fold changes in expression. Mouse *GAPDH* (P/N 4352662-0506003) was used as a control. Genes representative of each germinal layer were selected for analysis, i.e., *Lhx1* (Mm00521776_m1), *Nkx2.5* (Mm00657783_m1), *Gata4* (Mm00484689_m1), *Myocd* (Mm00455051_m1), *Oct4* (Mm00658129_gH), *Fgf4* (Mm0000438917_m1), *Mef2c* (Mm01340839_m1), *Gsc* (Mm00650681_g1), *Sox17* (Mm00488363_m1), *Sox7* (Mm00776876_m1), *Flk1* (Mm00440099_m1), *Sox1* (Mm00486299_s1) and *Sox2* (Mm00488369_s1). Ingenuity Pathway Analysis (IPA) 6.0 was used to map prioritized interactions between Sox transcription factors and key cardiovascular developmental regulators. Focused *in silico* interrogation was employed to identify database-curated interactions. Iterations of the network growing algorithm uncovered interactions of

Sox transcription factors and their connections with the Flk-1/CXCR4-signaling axis. Documented gene relationships were deconvoluted into a format compatible for network mapping by Cytoscape 2.6.0. A streamlined framework focused on SDF1/CXCR4, VEGF/Flk-1 and Sox transcription factors was generated with Cytoscape 2.6.0 (Faustino et al, 2008; Arrell et al, 2008). Pathways within the streamlined network were reiteratively investigated using IPA 6.0 to determine significance-ranked priorities of present cascades within focused cardiovascular-associated, as well as global, ontological layers (Faustino and Terzic, 2008).

RESULTS

Cardiac specification from pluripotent programs

Pluripotent embryonic stem cells provide a renewable pool of clonal progenitors capable of spontaneous differentiation into multiple cell types, generating herein three germinal layer embryoid aggregates *in vitro* (Figure 1). By day 15 of differentiation, progenitor cells within the embryoid aggregate morphed into an embryo-like structure (Figure 1A) harboring a complex mixture of tissues that included contractile zones positive for early cardiac markers, i.e. cardiac transcription factor Mef2c, and the sarcomeric α -actinin protein (Figure 1B). As the heart is the first organ to develop during embryogenesis, cardiac specification was an early event characterized by transition from an undifferentiated phenotype devoid of cardiac markers (Figure 1C) into a progeny displaying mature sarcomeres typical for Mef2c-positive cardiomyocytes (Figure 1D). Spontaneous cardiogenesis was limited to focal areas, in up to half of the forming embryoid bodies (n=50), indicating successful maturation of genetic programs that determine cardiac specification.

Flk-1 expression precedes up-regulation cardiac transcription factor

The yield of cardiac progenitors is enriched by targeted selection using lineage-specific biomarkers (Nelson et al, 2008). In the first three days of *in vitro* differentiation, the expression profile of embryonic stem cells was characterized by the abundance of pluripotent markers, including *Oct-4* and *Fgf-4* (Figure 2). The expression profile dramatically evolved at day 5, with a burst of markers associated with gastrulating embryonic tissue that included the prototypic *Sox1*, *Sox7*, *Sox17*, *Gsc*, and *Lhx1* (Figure 2). Ultimately, by day 7, the cardiac transcription factors *Nkx2.5*, *Mef2c*, *Myocardin*, and *GATA-4* were induced, fully engaging cardiac commitment (Figure 2). The mesoderm-restricted marker *Flk-1* (tyrosine kinase VEGF receptor 2 or KDR) demonstrated a unique gene expression profile that peaked at day 6, immediately following gastrulation but preceding the characteristic expression of cardiac transcription factors (Figure 2). Gene expression correlated with Flk-1 protein expression (Figure 3A–C). Specifically, 46% of the stem cell progeny mixture demonstrated positive cell surface expression of Flk-1 by day 5 of differentiation (Figure 3B). This time frame coincides with the earliest phase of primordial cardiac maturation defined by amplification of the cardiopoietic stem cell pool, characterized by nuclear translocation of the cardiac transcription factor Mef2c prior to expression of the sarcomeric α -actinin protein (Figure 3D–F). Thus, the expression dynamics of *Flk-1* presages the onset of cardiogenesis.

Sorted Flk-1⁺ progenitors segregate progeny enriched with Sox7

Flow sorting of embryonic stem cell progeny according to Flk-1 cell surface expression at day 5 of differentiation splits cells into distinct populations that are positive or negative for biomarker identification (Figure 4A). Beyond the differential expression pattern of Flk-1, sorted populations displayed distinct gene expression profiles (Figure 4B). The Flk-1⁺ population expressed *Oct-4*, *Sox2*, and *Sox17* genes (Figure 4B) that correlate with markers of pluripotency, ectoderm and endoderm, respectively. In contrast, genes associated with Flk-1 protein expression, at day 5 of embryonic stem cell differentiation, included the canonical cardiac transcription factors *Nkx2.5*, *GATA-4*, and *Myocardin*, enriched up to 5-fold in Flk-1

positive progenitors compared to Flk-1 negative counterparts (Figure 4B). Notably, *Sox7*, a member of the F-group of the Sox transcription family, demonstrated a 20-fold enrichment in Flk-1⁺ compared to Flk-1⁻ progenitors (Figure 4B). *Sox7* thus emerges as a potential marker of the fate of Flk-1⁺ progenitors.

Divergent *Sox7* expression in Flk-1 subpopulations

Flk-1⁺ cells isolated from embryonic stem cell progeny contain multi-lineage cardiovascular potential (Yang et al, 2008; Kattman et al, 2006; Baba et al, 2007), and recently biomarkers have been identified to enrich cardiac progenitors from the parental pool (Nelson et al, 2008). Embryonic stem cell derived progeny were here sorted according to tandem Flk-1 and CXCR4 cell surface expression after 5 days of *in vitro* differentiation (Figure 5A). The Flk-1⁺/CXCR4⁺ subpopulation, which segregates the cardiogenic potential of Flk-1 progeny (Figure 5B), expressed significantly lower levels of *Sox7*, compared to Flk-1⁺/CXCR4⁻ subpopulation (Figure 5C) that enrich for endothelial and smooth muscle markers of vasculogenesis (Nelson et al, 2008). The differential expression profile of *Sox7* was distinct from *Sox17*, a family member that demonstrated equivalent expression in Flk-1⁺/CXCR4⁺ and Flk-1⁺/CXCR4⁻ subpopulations (Figure 5C). Thus, a divergent *Sox7* expression profile is associated with lineage specification of Flk-1 progenitors at this early stage of differentiation.

Wnt/ β -catenin axis networks Flk-1/CXCR4 with Sox

Bioinformatic mining unearthed a framework of curated interactions that integrated *Sox7* along with other members of the F-group family of Sox transcription factor, namely *Sox17* and *Sox18*, with the cardiovascular SDF1/CXCR4 and VEGF/Flk-1(KDR) signaling axes (Figure 6). Among globally significant pathways embedded within this targeted framework, Wnt/ β -catenin signaling emerged as a prioritized functional cascade ($p=7.24 \times 10^{-6}$) that uniquely encompassed *Sox7*, *Sox17*, and *Sox18*. Further insight into the overall network propensities was provided by resolution of “Top Network Functions”. These discrete ontological indicators served as composite metrics to prioritize the parent ontology based on specific sub-functions according to a p-value ranking. “Cardiovascular Development” emerged as a primary function within the parental network ($p=4.24 \times 10^{-8}$), followed by “Organismal Development” ($p=2.69 \times 10^{-7}$) and “Cellular Movement” ($p=6.85 \times 10^{-7}$). Network removal of *Sox7* demoted “Cardiovascular Development”, while exhibiting minimal to no effects on remaining classifications. Categorical dissection of sub-functions comprising “Cardiovascular Development”, after *in silico Sox7* deletion, revealed diminution in significance of terms encompassing vascular formation, e.g., “Angiogenesis”, “Vascularization”, “Vasculogenesis”. Thus, bioinformatic mining reveals a significant predilection for Sox-derived control, potentially through the Wnt/ β -catenin axis, of multi-lineage cardiovascular differentiation of Flk-1⁺ stem cell progeny.

DISCUSSION

A priority in harnessing the regenerative potential of embryonic stem cells mandates selection of tissue-restricted progenitors, yet lack of predictive biomarkers remains a limitation. Integrating gene expression profiling, *ex embryo* validation, and bioinformatic deconvolution provided here an enabling platform to map pathways associated with lineage-restricted differentiation of stem cell progeny. The identified linkage between kinetics of the vascular endothelial growth factor (VEGF) receptor Flk-1 expression and the transition from pluripotency to lineage commitment establishes a traceable cell surface tag for efficient identification of stage-specific progenitor cells. Pre-cardiac progenitors, sorted from pro-vascular cohorts by Flk-1⁺ precursors which co-express CXCR4, enabled identification of the transcription factor *Sox7* as prognostic for programs within cardiovascular lineage specification. Specifically, bioinformatic dissection revealed developmental priority

reorganizations contingent on Sox7, in particular, with demotion of global cardiovascular development or diminution of vascular subfunctions. Thus, within a pool of heterogeneous subpopulations that share the VEGF/Flk-1 signaling axis, Sox7 emerges as a fate-modifying cofactor that distinguishes differentiation potential of cells that express Flk-1 (Figure 7).

The tyrosine kinase cell surface receptor Flk-1 is a recognized docking site for the vascular endothelial growth factor (VEGF), implicated in broad mesoderm-derived development (Shalaby et al, 1995) and cardiopoiesis (Kattman et al, 2006; Faustino et al, 2008; Yang et al, 2008). Established in embryonic development as a marker of early mesoderm (Nishikawa et al, 1998), Flk-1 is further recognized as a candidate biomarker for selecting early cardiovascular progenitors during *in vitro* differentiation, with peak expression bridging gastrulation with nascent cardiogenesis. Flk-1⁺ cells displayed a unique gene expression profile indicative of pro-cardiovascular differentiation and devoid of pluripotent transcripts. The present findings are in line with the recently established linkage of Flk-1 and cardiovascular differentiation potential (Garry and Olson, 2006; Baba et al, 2007; Yang et al, 2008), further verified herein by the transient amplification of the cardiac progenitor pool that coincided with maximal induction of the biomarker expression. Indeed, treatment of embryonic stem cell progeny with Flk-1 agonists stimulates increased rates of cardiogenesis from multi-lineage pools of progenitor cells (Chen et al, 2006; Faustino et al, 2008).

A distinctive feature within the Flk-1⁺ compared to the Flk-1⁻ gene expression profile was prominent enrichment in Sox7, a member of the F-group Sox family genes. Sox genes encode transcriptional factors with DNA-binding activity, mediated by the characteristic SRY-type HMG box motif responsible for sequence-specific DNA binding (Taniguchi, 1999). Sox7 is an essential element in non-mammalian cardiovascular development (Zhang et al, 2005). The F-group of Sox family transcription factors integrates Nodal and Wnt signaling pathways, and in model systems efficiently drives *de novo* cardiogenesis with ectopic overexpression in non-cardiac fields inducing cardiovascular lineage development (Zhang et al, 2005). Expression of Sox7 is required for proper regulation of mammalian vascular development (Cermenati et al, 2008; Pendeveille et al, 2008), and was identified by high-throughput analysis to be an essential transcription component for vascular differentiation in Flk-1 positive embryonic stem cell progeny (Yamauchi et al, 2007). Thus, Sox7 expression in stem cells is qualified to regulate the transcription of target genes and thereby dictate cell fate of cardiovascular progenitors.

The Sox family of transcription factors modulates signaling processes during differentiation (Lefebvre et al, 2007), in addition to directly regulating tissue-specific genes. Bioinformatic resolution of a network that encompasses the F-group of Sox transcription factors, and the VEGF cascade, revealed an embedded, highly prioritized Wnt/ β -catenin signaling axis, whose ability to drive early progenitor cells into mature cardiomyocytes has been independently validated (Faustino et al, 2008). In addition to its cardiomyogenic capacity, activated Wnt/ β -catenin signaling may function as a biphasic arbiter of cardiac and/or vascular fate selection during early embryonic development (Naito et al, 2006). In this study, differential Sox7 expression in the heterogeneous, gastrulating-equivalent pool of Flk-1⁺ progenitors was linked to cardiovascular lineage specification. This data was consistent with the reported ability of Sox7 to inhibit the Wnt/ β -catenin axis (Takash et al, 2001), associated with early stages of vasculogenesis and hematopoiesis (Naito et al, 2006). Wnt/ β -catenin signaling thus emerges as a putative coordinator for Sox7-mediated regulation, with a gatekeeper function for Flk-1⁺ progenitors that determines cardiovascular differentiation potential.

Together, this study demonstrates a biomarker-based approach that offers prediction and selection of a specific progenitor phenotype harboring pre-defined lineage-specific genetic programs from a heterogeneous pool of embryonic stem cell progenitors. Through this selective process, the pluripotent capacity of the embryonic stem cell source can be harnessed to

predictably execute tissue-restricted differentiation while minimizing uncontrolled growth. In this way, selection by Flk-1 isolates a mixture of cardiovascular progenitors with divergent expression profiles of *Sox7* and provides a paradigm for refining the regenerative therapeutic armamentarium from naïve embryonic stem cells to lineage-restricted cytotypes.

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References

- Arrell DK, Niederländer NJ, Faustino RS, Behfar A, Terzic A. Cardioinductive network guiding stem cell differentiation revealed by proteomic cartography of tumor necrosis factor alpha-primed endodermal secretome. *Stem Cells* 2008;26:387–400. [PubMed: 17991915]
- Baba S, Heike T, Yoshimoto M, Umeda K, Doi H, Iwasa T, Lin X, Matsuoka S, Komeda M, Nakahata T. Flk1⁺ cardiac stem/progenitor cells derived from embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model. *Cardiovasc Res* 2007;76:119–131. [PubMed: 17560966]
- Behfar A, Hodgson DM, Zingman LV, Perez-Terzic C, Yamada S, Kane GC, Alekseev AE, Puceat M, Terzic A. Administration of allogenic stem cells dosed to secure cardiogenesis and sustained infarct repair. *Ann NY Acad Sci* 2005;1049:189–198. [PubMed: 15965118]
- Behfar A, Perez-Terzic C, Faustino RS, Arrell DK, Hodgson DM, Yamada S, Puceat M, Niederlander N, Alekseev AE, Zingman LV, Terzic A. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. *J Exp Med* 2007;204:405–420. [PubMed: 17283208]
- Behfar A, Terzic A. Cardioprotective repair through stem cell-based cardiopoiesis. *J Appl Physiol* 2007;103:1438–1440. [PubMed: 17641218]
- Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A, Puceat M. Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J* 2002;16:1558–1566. [PubMed: 12374778]
- Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet* 2005;6:826–835. [PubMed: 16304598]
- Cermenati S, Moleri S, Cimbri S, Corti P, Del Giacco L, Amodeo R, Dejana E, Koopman P, Cotelli F, Beltrame M. Sox18 and Sox7 play redundant roles in vascular development. *Blood* 2008;111:2657–2666. [PubMed: 18094332]
- Chen Y, Amende I, Hampton TG, Yang Y, Ke Q, Min JY, Xiao YF, Morgan JP. Vascular endothelial growth factor promotes cardiomyocyte differentiation of embryonic stem cells. *Am J Physiol Heart Circ Physiol* 2006;291:H1653–1658. [PubMed: 16699073]
- Chung S, Dzeja PP, Faustino RS, Perez-Terzic C, Behfar A, Terzic A. Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. *Nat Clin Pract Cardiovasc Med* 2007;4:S60–67. [PubMed: 17230217]
- Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: The scientific foundations of cardiac repair. *J Clin Invest* 2005;115:572–583. [PubMed: 15765139]
- Faustino RS, Behfar A, Perez-Terzic C, Terzic A. Genomic chart guiding embryonic stem cell cardiopoiesis. *Genome Biol* 2008;9:R6. [PubMed: 18184438]
- Faustino RS, Terzic A. Interactome of a cardiopoietic precursor. *J Cardiovasc Trans Res* 2008;1:120–126.
- Foley A, Mercola M. Heart induction: embryology to cardiomyocyte regeneration. *Trends Cardiovasc Med* 2004;14:121–125. [PubMed: 15121161]
- Garry DJ, Olson EN. A common progenitor at the heart of development. *Cell* 2006;127:1101–1104. [PubMed: 17174889]

- Hidaka M, Stanford WL, Bernstein A. Conditional requirement for the Flk-1 receptor in the in vitro generation of early hematopoietic cells. *Proc Natl Acad Sci USA* 1999;96:7370–7375. [PubMed: 10377421]
- Hodgson DM, Behfar A, Zingman LV, Kane GC, Perez-Terzic C, Alekseev AE, Puceat M, Terzic A. Stable benefit of embryonic stem cell therapy in myocardial infarction. *Am J Physiol Heart Circ Physiol* 2004;287:H471–479. [PubMed: 15277190]
- Kattman SJ, Huber TL, Keller GM. Multipotent Flk-1⁺ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* 2006;11:723–732. [PubMed: 17084363]
- Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM, Sasse P, Rubenchik O, Fries JW, Wenzel D, Geisen C, Xia Y, Lu Z, Duan Y, Kettenhofen R, Jovinge S, Bloch W, Bohlen H, Welz A, Hescheler J, Jacobsen SE, Fleischmann BK. Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. *J Exp Med* 2006;203:2315–2327. [PubMed: 16954371]
- Laflamme MA, Murry CE. Regenerating the heart. *Nature Biotechnol* 2005;23:845–856. [PubMed: 16003373]
- Latif S, Masino A, Garry DJ. Transcriptional pathways direct cardiac development and regeneration. *Trends Cardiovasc Med* 2006;16:234–240. [PubMed: 16980180]
- Lefebvre V, Dumitriu B, Penzo-Méndez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. *Int J Biochem Cell Biol* 2007;39:2195–2214. [PubMed: 17625949]
- Martin-Puig S, Wang Z, Chien KR. Lives of a heart cell: Tracing the origins of cardiac progenitors. *Cell Stem Cell* 2008;2:320–331. [PubMed: 18397752]
- Menard C, Hagege AA, Agbulut O, Barro M, Morichetti MC, Brasselet C, Bel A, Messas E, Bissery A, Bruneval P, Desnos M, Puceat M, Menasche P. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. *Lancet* 2005;366:1005–1012. [PubMed: 16168783]
- Menasche P. The potential of embryonic stem cells to treat heart disease. *Curr Opin Mol Ther* 2005;7:293–299. [PubMed: 16121694]
- Murry CE, Field LJ, Menasche P. Cell-based cardiac repair. *Circulation* 2005;112:3174–3183. [PubMed: 16286608]
- Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, Komuro I. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci USA* 2006;103:19812–19817. [PubMed: 17170140]
- Nelson TJ, Ge ZD, Van Orman J, Barron M, Rudy-Reil D, Hacker TA, Misra R, Duncan SA, Auchampach JA, Lough JW. Improved cardiac function in infarcted mice after treatment with pluripotent embryonic stem cells. *Anat Rec A Discov Mol Cell Evol Biol* 2006;288:1216–1224. [PubMed: 17004246]
- Nelson TJ, Faustino RS, Chiriac A, Crespo-Diaz R, Behfar A, Terzic A. CXCR4⁺/FLK-1⁺ biomarkers select a cardiopoietic lineage from embryonic stem cells. *Stem Cells* 2008;26:1464–1473. [PubMed: 18369102]
- Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1⁺VE-cadherin⁺ cells at a diverging point of endothelial and hemopoietic lineages. *Development* 1998;125:1747–1757. [PubMed: 9521912]
- Olson EN. Gene regulatory networks in the evolution and development of the heart. *Science* 2006;313:1922–1927. [PubMed: 17008524]
- Passier R, van Laake LW, Mummery CL. Stem cell-based therapy and lessons from the heart. *Nature* 2008;453:322–329. [PubMed: 18480813]
- Perez-Terzic C, Behfar A, Mery A, van Deursen JM, Terzic A, Puceat M. Structural adaptation of the nuclear pore complex in stem cell-derived cardiomyocytes. *Circ Res* 2003;92:444–452. [PubMed: 12600892]
- Perez-Terzic C, Faustino RS, Boorsma B, Arrell DK, Niederlander NJ, Behfar A, Terzic A. Stem cells transform into a cardiac phenotype with remodeling of the nuclear transport machinery. *Nat Clin Pract Cardiovasc Med* 2007;4:S68–76. [PubMed: 17230218]

- Pendeville H, Winandy M, Manfroid I, Nivelles O, Motte P, Pasque V, Peers B, Struman I, Martial JA, Voz ML. Zebrafish Sox7 and Sox18 function together to control arterial-venous identity. *Dev Biol* 2008;317:405–416. [PubMed: 18377889]
- Sachinidis A, Fleischmann BK, Kolossov E, Wartenberg M, Sauer H, Hescheler J. Cardiac specific differentiation of mouse embryonic stem cells. *Cardiovasc Res* 2003;58:278–291. [PubMed: 12757863]
- Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66. [PubMed: 7596435]
- Singla DK, Hacker TA, Ma L, Douglas PS, Sullivan R, Lyons GE, Kamp TJ. Transplantation of embryonic stem cells into the infarcted mouse heart: formation of multiple cell types. *J Mol Cell Cardiol* 2006;40:195–200. [PubMed: 16288779]
- Shrivastava D, Ivey KN. Potential of stem-cell-based therapies for heart disease. *Nature* 2006;441:1097–1099. [PubMed: 16810246]
- Takash W, Cañizares J, Bonneaud N, Poulat F, Mattéi M, Jay P, Berta P. SOX7 transcription factor: Sequence, chromosomal localization, expression, transactivation and interference with Wnt signalling. *Nucleic Acids Res* 2001;29:4274–4283. [PubMed: 11691915]
- Taniguchi K, Hiraoka Y, Ogawa M, Sakai Y, Kido S, Aiso S. Isolation and characterization of a mouse SRY-related cDNA, mSox7. *Biochim Biophys Acta* 1999;1445:225–231. [PubMed: 10320775]
- van Laake LW, Van Hoof D, Mummery CL. Cardiomyocytes derived from stem cells. *Ann Med* 2005;37:499–512. [PubMed: 16278163]
- van Laake LW, Hassink R, Doevendans PA, Mummery C. Heart repair and stem cells. *J Physiol* 2006;577:467–478. [PubMed: 17008381]
- Wu SM, Chien KR, Mummery C. Origins and fates of cardiovascular progenitor cells. *Cell* 2008;132:537–543. [PubMed: 18295570]
- Yamada S, Nelson TJ, Crespo-Diaz RJ, Perez-Terzic C, Liu XK, Miki T, Seino S, Behfar A, Terzic A. Embryonic stem cell therapy of heart failure in genetic cardiomyopathy. *Stem Cells* 2008;000:00–000.
- Yamauchi F, Okadab M, Katoa K, Jaktb LM, Iwata H. Array-based functional screening for genes that regulate vascular endothelial differentiation of Flk1-positive progenitors derived from embryonic stem cells. *Biochim Biophys Acta* 2007;1770:1085–1097. [PubMed: 17532144]
- Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. Human cardiovascular progenitor cells develop from a KDR⁺ embryonic-stem-cell-derived population. *Nature* 2008;453:524–528. [PubMed: 18432194]
- Zhang C, Basta T, Klymkowsky MW. SOX7 and SOX18 are essential for cardiogenesis in *Xenopus*. *Dev Dyn* 2005;234:878–891. [PubMed: 16193513]

Figure 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 three 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 μ m. (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 μ m. **(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 μ m.

Figure 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*.

Figure 3.

Cell surface expression of Flk-1 identifies a transient population of progenitor cells at the stage of gastrulation. (A–C) Flow sorting at sequential stages of spontaneous differentiation of embryonic stem cells at days 3, 5, and 7 demonstrate a transient burst of Flk-1 positive progenitor cells that included 46% of the total cell population (n=3). (D–F) The cardiopoietic phenotype is derived within the first 7-days of differentiation. (D) Embryonic stem cells labeled with DAPI at day 3 lacked Mef2c or α -actinin expression. Bar, 3 μ m. (E) Cardiopoietic progenitors separated on density gradient express nuclear Mef2c (cyan) without sarcomeric α -actinin expression (red). Bar, 10 μ m. (F) Cardiomyocytes purified from embryonic stem progeny according to density isolation demonstrate robust α -actinin expression (red) along with nuclear localized Mef2c (cyan). Bar, 5 μ m.

Figure 4.

Embryonic stem cells sorted according to Flk-1 at the stage of gastrulation reveals Sox7 enrichment. **(A)** Embryonic stem cell progeny at day 5 were flow sorted in order to purify Flk-1⁺ from Flk-1⁻ populations. **(B)** Gene expression profiles of Flk-1⁺ and Flk-1⁻ populations were determined by RT-PCR, and comparison of relative expression levels identified. Genes with higher expression profiles in the Flk-1⁺ compared to the Flk-1⁻ population are plotted as positive values with negative values, indicating higher gene expression level in the Flk-1⁻ subpopulation. The Flk-1⁻ population expressed higher levels of the pluripotent marker *Oct4*, and non-mesodermal transcription factors *Sox2* and *Sox17*. The Flk-1⁺ population was enriched with *Sox7* expression and included higher levels of *Flk-1* and cardiogenic transcription factors *GATA4*, *Nkx2.5*, and *Myocardin*.

Figure 5.

Sox7 is depleted in the cardiogenic subpopulation of Flk-1⁺ progenitors. (A) Embryonic stem cell progeny sorted according to Flk-1 and CXCR4 expression after 5-days of differentiation demonstrate two distinct pools of Flk-1 cells without (Q1) and with CXCR4 expression (Q2). (B) Immunostaining for sarcomerogenesis, as indicated by α -actinin expression, demonstrates cardiogenesis in Flk-1⁺/CXCR4⁺ subpopulation of embryonic stem cell progeny. (C) RT-PCR analysis demonstrates relative expression levels between Flk-1⁺/CXCR4⁻ and Flk-1⁺/CXCR4⁺ that are significantly different for *Sox7* while being similar for *Sox17*. * P-value < 0.05.

Figure 6.

Bioinformatic mapping of Sox transcription factors, in the context of Flk-1/CXCR4 signaling, reveals a prioritized role for β-catenin. *Sox7*, *Sox17* and *Sox18* comprise the F-group of the Sox family of transcription factors (dark blue) that integrates with CXCR4 and Flk-1 cardiovascular signaling pathways (red and green) through β-catenin, prioritized among all signaling pathways associated with this streamlined framework. Corresponding p-value given.

Figure 7.

A model for cell fate determination of Flk-1 positive population that integrates cardiovascular progenitor cell differentiation with divergent *Sox7* expression. Flk-1 subpopulations that express increased levels of *Sox7* are associated with a vascular phenotype compared to a cardiogenic phenotype with reduced expression of *Sox7*.