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SDF-1-Enhanced Cardiogenesis Requires CXCR4 Induction in Pluripotent Stem Cells

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Abstract

Transformation of pluripotent stem cells into cardiac tissue is the hallmark of cardiogenesis, yet pro-cardiogenic signals remain partially understood. Preceding cardiogenic induction, a surge in CXCR4 chemokine receptor expression in the early stages of stem cell lineage specification coincides with the acquisition of pre-cardiac profiles. Accordingly, CXCR4 selection, in conjunction with mesoderm-specific VEGF type II receptor FLK-1 co-expression, segregates cardiogenic populations. To assess the functionality of the CXCR4 biomarker, targeted activation and disruption were here exploited in the context of embryonic stem cell-derived cardiogenesis. Implicated as a cardiogenic hub through unbiased bioinformatics analysis, induction of the CXCR4/SDF-1 receptor–ligand axis triggered enhanced beating activity in stem cell progeny. Gene expression knockdown of CXCR4 disrupted spontaneous embryoid body differentiation and blunted the expression of cardiogenic markers MEF2C, Nkx2.5, MLC2a, MLC2v, and cardiac-MHC. Exogenous SDF-1 treatment failed to rescue cardiogenic-deficient phenotype, demonstrating a requirement for CXCR4 expression in mediating SDF-1 effects. Thus, a pro-cardiogenic signaling role for the CXCR4/SDF1 axis is herein revealed within pluripotent stem cell progenitors, exposing a functional target to promote lineage-specific differentiation.

Keywords

Cardiogenesis; Pluripotent stem cells; SDF-1; CXCR4

Introduction

The innate regenerative capacity of the heart underlies continuous homeostatic replacement and cell turnover, yet is frequently inadequate to reestablish myocardial function following massive tissue loss in the setting of severe injury [1–4]. Pluripotent stem cell-based therapy can restore native tissue architecture and repair cardiac function, offering in principle a promising approach to boost the endogenous mechanisms of regeneration [5–7]. In this regard, understanding natural signaling pathways that drive cardio-genesis in embryonic development has facilitated the discovery of platforms for rational, de novo generation of extra-embryonic heart muscle [8, 9]. In this way, cardiogenic cells have recently been obtained via a flow-sorting strategy that extracted a progenitor subpopulation from differentiating pluripotent stem cell pools according to a stage-specific biomarker expression profile [10]. Specifically, a surge in CXCR4 expression during embryonic stem cell differentiation preceding cardiogenic induction enabled, in conjunction with the mesoderm marker Flk-1, selection of early pre-cardiac progenitors from an unrestricted population of embryoid body cytotypes [10]. Although biomarker-positive progenitors have been associated with the endogenous pro-cardiac developmental phenotype [11], the functional impact of the CXCR4 chemokine receptor in the context of stem cell-derived cardiogenesis remains largely unknown.

Correlating the dynamics of germinal lineage differentiation, maximal gene expression of CXCR4 coincides in embryoid bodies with the expression patterns that reflect gastrulating embryonic tissue [10]. As single biomarkers are commonly associated with multi-lineage precursors in the early stages of development and are thereby insufficient to definitively distinguish differentiation outcomes, the overlapping expression kinetics of CXCR4 with

that of Flk-1 provided a dual biomarker signature to sufficiently enrich a pro-cardiac cytotype [10]. While CXCR4 surface expression broadly identifies early mesendoderm lineages [12], Flk-1 is restricted to mesoderm derivatives excluding CXCR4-expressing endodermal cells [13, 14]. Indeed, co-expression of biomarkers preceded the induction of canonical cardiac transcription factors, suggesting a CXCR4-dependent predecessor to cardiac specification [10].

In embryogenesis, the biologic relevance of the CXCR4 receptor and its natural ligand stromal cell-derived factor 1 (SDF-1) is supported by the established role in which SDF-1 gradients initiate lineage-specific migration of CXCR4-positive progeny [15]. In fact, heart development has been consistently sensitive to the disruption of the CXCR4/SDF-1 axis with ventricular septum defects noted in homozygous null mutants for CXCR4 or SDF-1 [16, 17]. Moreover, in adult life, the CXCR4/SDF-1 axis provides critical signaling for cell homing and intra-tissue retention, and defective stem cell migration leads to myelokathexis, a human deficiency syndrome described in patients harboring CXCR4 mutations [18]. Conversely, pharmacologic modulation of CXCR4/SDF-1 axis has become routine practice to augment hematopoietic stem cell recruitment to the peripheral blood in preparation for bone marrow stem cell transplantation [19] and has provided therapeutic advantages in assisting in tissue repair following hypoxic injuries [20]. Specifically in the adult heart, expression of SDF-1 is induced by hypoxia and provides a principal component of the stress signal for migratory cells, such as macrophages, leukocytes, and tissue-specific progenitors [21–23]. With its unique chemotactic propensity, CXCR4/SDF-1 interaction may provide a relevant target to not only promote progenitor cell localization to the most appropriate niche environment but also directly contribute to cardiac-specific differentiation within the injured heart [24]. While CXCR4/SDF-1 signaling pairs are linked to natural heart development and presumed regenerative mechanisms, the actual requirement for the induction of CXCR4 in pluripotent stem cells has remained untested in the context of de novo cardiogenesis.

Here, CXCR4/SDF-1 signaling axis was found central in stem cell-derived cardiogenesis through bioinformatics analysis of transcriptome data sets, validated in pluripotent stem cell three-dimensional cultures by a direct demonstration of CXCR4-dependent acceleration of cardiac differentiation. Moreover, siRNA disruption of CXCR4 expression in the initial stages of differentiation adversely affected stem cell cardiogenesis in which exogenous SDF-1 supplementation was not sufficient to achieve rescue, indicating a required coupling of the SDF-1 ligand through available CXCR4 receptors during stem cell differentiation. Pro-cardiac signaling mediated by the CXCR4/SDF-1 axis provides therefore a previously unrecognized functional target to support lineage-specific differentiation of pluripotent stem cells.

Materials and Methods

Cardiogenic Induction from Pluripotent Stem Cells

The MHC-lacZ R1 embryonic stem cell line that expresses a cassette containing the MHC promoter upstream of β -galactosidase cDNA was used to monitor acquired cardiogenesis, as previously described [11]. Stem cells were maintained in Glasgow's Minimum Essential Medium (BioWhittaker-Cambrex) supplemented with pyruvate and L-glutamine (Cellgro), non-essential amino acids (Mediatech), β -mercaptoethanol (Sigma-Aldrich), 15% fetal calf serum (Invitrogen), and leukemia inhibitory factor (ESGRO, Chemicon). Embryoid bodies were generated according to the hanging drop method with or without treatment on day 4.5 with SDF-1 (100 ng/mL, provided by Dr. Brian Volkman, Medical College of Wisconsin, Milwaukee, WI).

Stable CXCR4 Gene Knockdown

Wild-type MHC-lacZ R1 embryonic stem cells were plated at a density of 100,000 cells/mL in serum-free Dulbecco's modified Eagle medium and incubated with lentivirus siRNA (L.K.O. vector, Open Biosystems). Four different CXCR4 siRNA clones were tested for efficiency in achieving gene knockdown and generating stable cell lines. After 12 h, cells were washed and incubated with normal propagation media (15% serum) for an additional 24 h to allow recovery. Wild-type embryonic stem cells and the four CXCR4 siRNA clones were subsequently treated with 4 ng/mL puromycin for 24 h. Antibiotic resistant cell lines were collected and propagated for cardiac differentiation.

RNA Isolation and Microarrays

Total RNA was isolated using a combination of gDNA Eliminator and RNeasy columns (Qiagen protocol). Three independent biological replicates were obtained for each condition, i.e., pluripotent embryonic stem cells (day 0) and unsorted embryoid body progeny (day 5). Double-stranded cDNA and labeled complementary cRNA were obtained from isolated RNA with the latter hybridized to the Mouse 430 2.0 GeneChip (Affymetrix). Gene Chips were scanned and data visualized using the Affymetrix Microarray Suite 5.0 software [11].

Gene Expression Analysis

Microarray gene expression raw data were MIAME compliant and were deposited with the Gene Expression Omnibus database (accession no. GSE20841). Analysis was performed using the GeneSpring GX 7.3.1 software (Agilent Technologies). All probe sets were filtered according to chip-specific background noise, and genes expressing signals below the threshold were removed. Probe sets were normalized to values in pluripotent stem cells. To ensure that only genes with significant transcriptional changes during embryonic stem cell differentiation were selected, all probe sets were filtered according to a flag value of Present or Marginal in at least two out of three replicates for each cellular phenotype. A filter on volcano plot was applied to identify significant changes in gene expression (>2-fold, $p < 0.05$) in day 5 cytotypes compared to pluripotent embryonic stem cells. Benjamini-Hochberg multiple testing correction was applied to extract a relevant gene pool for day 5 progeny.

Quantitative Gene Expression

cDNA was synthesized from total RNA samples using SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using a TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 50 μ L PCR reaction mixture included 3 μ L RT product, 25 μ L TaqMAN Universal Master Mix (Applied Biosystems), 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). The threshold cycle (Ct) was defined as the fractional cycle number at which fluorescence passes detection threshold. Ct values were subsequently converted into relative fold changes determined using the 2^{-CT} method, normalized to *Gapdh*. Genes representative for pluripotency (*Oct4*, *Sox2*), gastrulation (*Cxcr4*, *Sox17*, *Gsc*, *Lhx1*), and cardiogenesis (*Mesp1*, *Nkx2.5*, *Mef2c*, *MLC2a*, *MLC2v*, *-MHC*) were analyzed.

Bioinformatics Function Analysis

Transcriptional profiles were functionally analyzed using the Ingenuity Pathway Analysis Software IPA 8.0 (www.ingenuity.com). Based on the curated Ingenuity Knowledge Database and using a right-tailed Fisher's exact test, overrepresented functions and pathways associated with intersecting gene lists were identified and the significance of

association (p value) was calculated based on the probability of pathway assembly from a random set of genes of the same size as the input list [25].

X-gal Quantification of Cardiac MHC-Rich Areas

Embryoid bodies for each experimental condition were fixed with glutaraldehyde (Sigma-Aldrich) for 30 min at room temperature, followed by a 30-min wash with phosphate-based buffer and overnight incubation at 37°C with X-gal staining solution (Invitrogen), as previously described [11]. Embryoid body images were captured with a ProgRes C3 camera-equipped Zeiss stereo Discovery V20 microscope.

Statistics

Values are provided as mean $\pm$ SE, and Student's t tests with 95% confidence intervals were used to compare treatment groups. A $p < 0.05$ was predetermined as significant.

Results

Bioinformatics Interrogation Reveals CXCR4-Dependent Cardiogenic Pathway

Head-to-head comparison of transcriptomes extracted from pluripotent stem cells (Pluripotent) versus embryoid bodies differentiated toward gastrulation (Gastrulation) uncovered a divergent gene pool characterized by 560 differentially expressed transcripts (>2 -fold difference, $p < 0.05$, Benjamini–Hochberg multiple testing correction; Fig. 1a, left). CXCR4, linked to a cardiac phenotype (ventricular septal defect) and associated with an expression time course that precedes the induction of canonical cardiac transcription factors NKX2.5 and MEF2C [10], was identified on volcano plots among the highest upregulated transcripts at day 5 of differentiation (Fig. 1a, right). In silico knockout of CXCR4, from the pool of day 5 (Gastrulation) transcripts, altered developmental priorities by demoting “heart development,” without a detectable effect on other tissue-specific developmental functions that contain CXCR4-dependent components (Fig. 1b). Ingenuity pathway building algorithm mapped downstream signaling highways for the CXCR4/SDF-1 axis including JAK/STAT3, PI3K/AKT, and RAS/MAPK signaling (Fig. 1c). These putative intracellular effectors targeted a common final effect on the nucleus, regulating gene expression and inducing the expression of canonical cardiac transcription factors (Fig. 1c). Validating the functionality of the mapped CXCR4/SDF-1 network, treatment of differentiating stem cells with SDF-1 (100 ng/mL), the established ligand of the CXCR4 receptor, significantly accelerated the beating activity in embryoid bodies compared to untreated controls (Fig. 1d). Thus, transcriptome analysis unmasked a CXCR4-dependent cardiogenic pathway in pluripotent stem cell differentiation, with the CXCR4/SDF-1 axis sufficient to induce early cardiogenic specification and accelerate cardiogenic development.

Stable Integration of siRNA Prevents Stage-Specific CXCR4 Induction

The temporal expression profile of CXCR4 mRNA is characterized by a surge at day 5 of embryonic stem cell differentiation that coincides with the process of gastrulation (Fig. 2a). To silence CXCR4 expression, four siRNA constructs were constitutively expressed following drug-selectable enrichment in pluripotent stem cells. Two such clones (clones 2 and 3) demonstrated a significant down-regulation of CXCR4 mRNA at day 5 (Fig. 2b), and one clone (clone 3) displayed $<5\%$ cell surface expression of the CXCR4 receptor protein compared to $>20\%$ for wild-type controls (Fig. 2c). Accordingly, clone 3 maximally downregulated CXCR4 expression at both the mRNA and protein level and prevented the stage-specific induction of chemokine receptor during stem cell differentiation.

Cardiogenic Phenotype Abrogated with CXCR4 Silencing

Pluripotent stem cell lines containing CXCR4 siRNA constructs were able to aggregate and expand into embryoid bodies, initially maintaining a morphology comparable to wild-type controls. Yet, distinct differences became apparent with continued differentiation as clone 3 (CXCR4⁻) displayed impaired beating activity (only ~25% at day 8 compared to ~65% for wild type) and lack of myosin heavy chain-positive areas (Fig. 3). Compared to the wild-type phenotype that demonstrated robust expression of X-gal cardiac myosin heavy chain (α-MHC)-rich areas which corresponded to beating foci in live embryoid bodies [11], the cardiac-deficient phenotype characteristic for clone 3 (CXCR4⁻) lacked X-gal staining accompanied by a significant downregulation in the expression of cardiac transcription factors Nkx2.5, MEF2C, MLC2v, and α-MHC (Figs. 3 and 4). Therefore, decreased levels of CXCR4 during the first week of differentiation resulted in the abrogation of cardiac markers and suggested a delay or repression in stem cell-derived cardiogenesis.

SDF-1 Enhanced Cardiogenesis Requires CXCR4 Induction

Treatment with SDF-1, the ligand for the CXCR4 receptor, significantly augmented the cardiogenic yield, monitored as beating activity, from day 6 to day 8 of wild-type pluripotent stem cell-derived embryoid bodies (Fig. 5a). Silencing of CXCR4 with siRNA eliminated the SDF-1 augmentation of cardiogenicity (Fig. 5a). Similarly, stable integration of CXCR4 siRNA nullified the pro-cardiac action of SDF-1 on cardiac transcription factor MEF2c gene expression (Fig. 5b) and X-gal staining for the expression of the cardiac-specific α-MHC-lacZ transgene (Fig. 5c). Thus, SDF-1-enhanced cardiogenesis is dependent on the native expression levels of CXCR4, suggesting that the cardiogenic effect is not mediated by alternative mechanisms or related chemokine receptors.

Discussion

Complementing the innate regenerative capacity of the heart, extracardiac progenitor populations primed for stage-specific activation of cardiogenic traits are increasingly investigated as novel strategies in the setting of permanent tissue injury [26–30]. To this end, stem cell-derived progeny selected according to the CXCR4 biomarker, in combination with co-expression of the VEGF type II receptor FLK-1, has been recently demonstrated to be uniquely equipped for de novo cardiac differentiation [10]. Although cardiac progenitors have been successfully selected based on stage-specific biomarker expression, this study reveals for the first time that the CXCR4 receptor/SDF-1 ligand axis is functional in supporting and accelerating cardiogenesis from pluripotent stem cell pools. Coupled with the endogenous release of SDF-1 from injured heart tissue [31], CXCR4-positive progeny may thereby offer a specialized subpopulation derived from an unlimited supply of pluripotent sources to respond efficiently to injury and ensure targeted functional tissue regeneration.

Augmenting the appropriate cell type to meet in situ requirements of the injured myocardium provides a rational strategy to optimize a curative solution of stem cell-based therapy for specific disease conditions that lead to refractory heart failure [8, 9, 23, 26]. However, success of stem cell-based interventions is balanced by inherent technical limitations of existing differentiation platforms to provide high-yield homogeneous populations of pro-cardiac phenotypes and by mechanistic challenges of delivery to ensure engraftment at the intended tissue destination [32–35]. By resolving biomarkers associated with pre-cardiac migration and differentiation during natural embryogenesis, a CXCR4-selectable population of cardiac progenitors demonstrating chemotactic responsiveness toward the ischemic heart was identified [36]. Dissecting hierarchies that underlie lineage commitment extracted a prioritization of systems networks linked to cardiogenic

specification [10]. Here, CXCR4 was confirmed as a significantly upregulated gene in early differentiation and linked to cardiac gene expression through the STAT, AKT, and MAPK pathways. Moreover, ablating CXCR4 in silico reprioritized developmental functions, demoting cardiovascular development under CXCR4-null conditions. Thus, disruption of CXCR4 by bioinformatics means predicted a necessary role for the chemokine receptor in stem cell-derived cardiogenesis.

Indeed, exogenous SDF-1 supplementation during the initial stages of stem cell differentiation here promoted cardiogenic outcome with increased beating activity. Conversely, gene expression silencing of the CXCR4 chemokine receptor in embryonic stem cells abrogated cardiogenicity. The siRNA constructs applied herein were constitutively expressed in pluripotent stem cells and demonstrated variable knockdown levels of endogenous CXCR4 receptor as monitored by the peak expression levels at day 5 of differentiation. The most dramatic effect on beating activity was noted in the clone with the highest knockdown of CXCR4 expression at the mRNA and protein levels. Disruption of CXCR4 expression notably affected normal embryoid body formation containing cardiac progenitors, as shown by a decreased expression of cardiac transcription factors MEF2C, Nkx2.5, MLC2a, MLC2v, and cardiac-MHC. Thus, interference with CXCR4 expression had a negative impact on the downstream gene expression profile indicating a direct linkage to the early mechanisms of cardiogenesis, validating the prediction of unbiased network analysis.

The CXCR4/SDF-1 axis was traditionally considered unique in that the SDF-1 ligand had only a single receptor. Since SDF-1 could also signal through the related CXCR7 receptor, as revealed more recently [36, 37], exogenous SDF-1 was tested on CXCR4 knockdown clones. These experiments demonstrated that SDF-1 was reproducibly sufficient to accelerate beating activity and cardiac gene expression in wild-type controls. Yet, the cardiogenesis-deficient phenotype in the absence of natural CXCR4 expression was not rescued by exogenous SDF-1 supplementation, suggesting that an intact CXCR4 receptor is necessary for the pro-cardiac stimulatory action of SDF-1. These findings suggest active signaling through the CXCR4/SDF-1 axis during cardiogenesis, with the CXCR4 chemokine receptor serving not only as a passive biomarker but also as an active, functional signaling participant during heart specification. Although these data do not exclude other receptors, such as CXCR7, from contributing to SDF-1 augmentation of cardiogenesis, the results provide a direct evidence that CXCR4 is an essential component either independently or possibly through heterodimerization with other chemokine receptors.

Initial evidence for a role of CXCR4/SDF-1 during cardiogenesis was postulated by the observed ventricular septal defect in knockout mice [16, 17] and by the role of CXCR4 surface expression, together with FLK-1, uniquely identifying cardiogenic precursors from embryonic stem cells [10]. Moreover, circulating bone-marrow-derived CXCR4-positive progenitors may enable site-specific localization to the SDF-1-secreting ischemic myocardium [38], and emerging evidence suggests that subpopulations of stem cells expressing CXCR4 have favorable characteristics for cardiac regeneration [39]. With its unique pro-cardiogenic and chemotactic propensity, CXCR4/SDF-1 interaction may provide a relevant target to not only promote progenitor cell localization to the most appropriate niche environment but also significantly contribute to cardiac-specific differentiation within the injured heart.

Conclusion

The CXCR4/SDF-1 axis stimulates cardiogenesis from pluripotent stem cell backgrounds. This effect is dependent on the CXCR4 chemokine receptor as CXCR4⁻ knockdown

embryoid bodies demonstrate deficient cardiac lineage specification and reduced cardiogenic yield, which cannot be rescued by the exogenous administration of SDF-1. Overall, this study demonstrates a novel role for the CXCR4/SDF-1 axis, with CXCR4 being a functional signaling molecule during cardiac specification, as opposed to a passive biomarker, that is necessary for SDF-1 to augment de novo cardiogenesis from pluripotent stem cells.

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Fig. 1.

Induction of the CXCR4/SDF-1 axis promotes cardiogenesis. **a** Transcriptome comparison of embryonic stem cells prior to differentiation (*Pluripotent*) and as day 5 differentiated embryoid bodies (*Gastrulation*) demonstrates a divergent gene pool of 560 transcripts (>2-fold difference, $p < 0.05$, Benjamini–Hochberg multiple testing correction). **b** In silico knockout of CXCR4 altered developmental priorities by demoting “heart development” without disrupting other tissue-specific developmental functions. **c** Ingenuity pathway analysis mapped downstream signaling highways for the CXCR4/SDF-1 axis which includes JAK/STAT3, PI3K/AKT, and RAS/MAPK signaling capable of driving cardiac gene expression. **d** SDF-1 treatment (100 ng/mL) significantly accelerated the initial beating activity in embryoid bodies compared to untreated controls

Fig. 2. CXCR4 knockdown blunts the induction of chemokine expression. **a** Stage-specific expression of CXCR4 peaks after 5 days of differentiation. **b** Of the four siRNA clones (1–4) constitutively expressed in pluripotent stem cells, clones 2 and 3 abrogated endogenous CXCR4 expression of mRNA at day 5. **c** Clone 3 also reduced cell surface expression of CXCR4 from 22% in parental cells to 5% at day 5 of differentiation

Fig. 3. Disruption of CXCR4 induction prevents spontaneous beating activity. Spontaneous differentiation from pluripotent stem cells in embryoid bodies allows the formation of cardiac tissue. Beating activity, an indicator of cardiogenesis, increases between days 6 and 8 of differentiation in control cells. Clone 3 significantly decreased beating activity and cardiac gene expression throughout the early stages of differentiation compared to other siRNA clones despite having little impact on the cell growth of the embryoid body

Fig. 4.

Cardiogenic gene expression dependent on CXCR4 expression. Wild-type embryoid bodies (*Control*) differentiate into three-dimensional clusters that contain cardiac-like tissue expressing α -MHC/lacZ reporter transgene after 7 days of differentiation. In contrast, CXCR4⁻ embryoid bodies demonstrate an absence of α -MHC despite expanding as normal morphological clusters at day 7. CXCR4 knockdown was also accompanied by a significant down regulation in cardiac transcription factors, such as Nkx2.5, MEF2C, MLC2v, and α -MHC

Fig. 5.

Treatment with SDF-1 is not sufficient to promote cardiogenesis in the absence of CXCR4. Embryoid bodies treated with exogenous SDF-1 demonstrate a significant induction of cardiogenic pathways. CXCR4^{-/-} embryoid bodies (clone 3) lacked spontaneous cardiogenesis as documented according to beating activity (a) and cardiac gene expression indicated by endogenous MEF2C (b). Exogenous treatment with SDF-1 was unable to rescue deficiencies in cardiogenesis. c Embryoid bodies demonstrated a lack of X-gal staining for expression of the cardiac-specific transgene, -MHC-lacZ construct in the clone 3 knockdown that remained unaffected by SDF-1 treatment