Misregulation of SDF1-CXCR4 Signaling Impairs Early Cardiac Neural Crest Cell Migration Leading to Conotruncal Defects

Sophie Escot, Cédrine Blavet, Sonja Härtle, Jean-Loup Duband, Claire Fournier-Thibault

Rationale: Cardiac neural crest cells (NCs) contribute to heart morphogenesis by giving rise to a variety of cell types from mesenchyme of the outflow tract, ventricular septum, and semilunar valves to neurons of the cardiac ganglia and smooth muscles of the great arteries. Failure in cardiac NC development results in outflow and ventricular septation defects commonly observed in congenital heart diseases. Cardiac NCs derive from the vagal neural tube, which also gives rise to enteric NCs that colonize the gut; however, so far, molecular mechanisms segregating these 2 populations and driving cardiac NC migration toward the heart have remained elusive.

Objective: Stromal-derived factor-1 (SDF1) is a chemokine that mediates oriented migration of multiple embryonic cells and mice deficient for Sdf1 or its receptors, Cxcr4 and Cxcr7, exhibit ventricular septum defects, raising the possibility that SDF1 might selectively drive cardiac NC migration toward the heart via a chemotactic mechanism.

Methods and Results: We show in the chick embryo that Sdf1 expression is tightly coordinated with the progression of cardiac NCs expressing Cxcr4. Cxcr4 loss-of-function causes delayed migration and enhanced death of cardiac NCs, whereas Sdf1 misexpression results in their diversion from their normal pathway, indicating that SDF1 acts as a chemoattractant for cardiac NCs. These alterations of SDF1 signaling result in severe cardiovascular defects.

Conclusions: These data identify Sdf1 and its receptor Cxcr4 as candidate genes responsible for cardiac congenital pathologies in human. (Circ Res. 2013;113:505-516.)

Key Words: cardiac development ■ cardiac neural crest ■ conotruncal defects genes ■ heart defects, congenital ■ receptors, CXCR4 ■ migration ■ SDF1 chemokine

Heart formation involves different cell populations originating from distinct embryonic territories. The first cardiogenic cells derive from the cardiac crescent known as primary heart field. They form the primitive cardiac tube and ultimately provide the left ventricle, the atrioventricular canal, and part of the atria. The second cardiogenic population appears later in the second heart field. It is incorporated into the primitive heart via the arterial pole and contributes to most of the right ventricle, the conus, the truncus, and also the rest of the atria. Cardiac neural crest cells (NCs), originating from the hindbrain, transit through the pharyngeal arches and associate with the second heart field before entering the heart. They play a major role in the outflow tract remodeling and contribute to the septa and valves, allowing separation of the pulmonary trunk from the aorta. Ablation of cardiac NCs in chick results in severe heart defects, such as defective outflow septation, outflow misalignment, and mispatterning of the great arteries. In addition, cardiac NC ablation disrupts development of the arterial pole by interfering with the second heart field integration into the heart. These anomalies mirror those found in congenital heart diseases, including ventricular septal defects (VSD), persistent truncus arteriosus (PTA), double-outlet right ventricle (DORV), tetralogy of Fallot, as well as the DiGeorge and velo-cardial-facial syndromes.

The prospective cardiac NC territory has been mapped using chick-quail chimeras to the axial region situated between the otic placode and the third somite, thereafter called cardiac level. This domain overlaps the vagal neural tube that spans the first 7 somites and from which also derive the NC progenitors of the enteric ganglia, vagus nerve, superior cervical ganglion, as well as cartilaginous, supportive, and endocrine tissues of the neck. Given the diversity of cell types arising from NCs in this region, one important question concerns how these populations are segregated and selectively driven to their final destinations. Cardiac NCs undergo migration away from the neural tube by following a dorsolateral path under the ectoderm, leading them to the pharyngeal arches. After pausing, they resume migration under the pharyngeal endoderm and,
along the aortic arch arteries, populate the cardiac outflow tract where they contribute the aorticopulmonary septum, and ultimately colonize the heart to form the ventricular septum and cardiac ganglia.\(^3\) Contrary to cardiac NCs, enteric NCs and progenitors of the peripheral nervous system of the neck follow a ventral route through the somites to reach the pharynx,\(^{10}\) suggesting that cardiac NCs are segregated precociously from the other NC populations by specific guidance cues.

Chemoataxis may account for the precision by which NCs reach their targets. The recent discoveries that the stromal-derived factor-1 (SDF1) chemokine may drive migration of some NC populations,\(^{11–13}\) that mice deficient for Sdf1 or its receptors, Cxcr4 and Cxcr7, exhibit heart septal defects\(^{15–16}\) suggest that SDF1 signaling may contribute to the guidance of cardiac NCs to the heart. Here, we show in the chick embryo that Sdf1 misexpression results in divergent spatiotemporal patterns in the ectoderm along their migration route. Cxcr4 signaling targets specific guidance cues. First, NCs express Cxcr4 during their initial migration to the pharyngeal arches, and that Sdf1 expression correlates with cardiac NC migration along their migration route. Cxcr4 loss-of-function causes delayed migration and enhanced death of cardiac NCs, whereas Sdf1 misexpression results in diversions of NCs from their normal migration pathways but none of them affect enteric NCs, indicating that SDF1 acts as a chemoattractant specific for cardiac NCs. Finally, alterations of SDF1 signaling during cardiac NC migration lead to severe cardiovascular defects, demonstrating that SDF1 signaling through Cxcr4 is required for correct heart morphogenesis. These data open new avenues for identifying genes implicated in cardiac congenital pathologies in human.

**Methods**

SDF1 Signaling Correlates With Cardiac NC Migration to the Heart

To determine whether SDF1 might constitute a guidance cue for cardiac NCs, we first analyzed in the chick embryo the expression profiles of Sdf1, Cxcr4, and Cxcr7 during their migration to the heart. NCs emerge from the cardiac neural tube at Hamburger and Hamilton\(^{17}\) (HH) stage 10 (ie, embryonic day [E] 1.5) and can be identified because of HNK1 or Sox10 markers. Sdf1 that was not detectable at the cardiac level before NC migration (Online Figure I) became prominent at the onset of migration in the ectoderm situated on either side of the neural tube (Figure 1A and 1C).

Coincidentally, Cxcr4 was expressed specifically in NCs emigrating from the cardiac neural tube and invading the dorsolateral pathway. Simultaneous detection of Sdf1 and Cxcr4 revealed strikingly coordinated spatiotemporal patterns in the ectoderm and underlying NCs throughout migration. As NCs progressed toward the circumpharyngeal region, Sdf1 expression was progressively reduced to the lateral ectoderm, abutting at the front of the Cxcr4-positive NCs and, at stage HH14 (E2), it was restricted to a discrete portion of the ventrolateral ectoderm along pausing NCs (Figure 1B, 1D, and 1E). It is noteworthy that although Sdf1 and Cxcr4 were prominent at the cardiac level, they were also found in other locations, for example, the fore and midbrain, as well as in other NC populations, for example, those emerging from rhombomere-4 (Online Figure I). However, Sdf1 expression in the ectoderm was limited to the cardiac level and was not apparent more caudally beyond the third somite, and vagal NCs migrating ventrally showed no Cxcr4 or Cxcr7 expression (Online Figure I). In contrast to Cxcr4, Cxcr7 expression was never observed in NCs during their subectodermal migration to the pharyngeal arches (Online Figure I).

At E2.5, cardiac NCs resume migration under the pharynx. Because both Sox10 and HNK1 are lost by NCs in the pharyngeal arches after HH14 and that HNK1 becomes instead expressed in the second heart field developing along the endoderm,\(^{20}\) we investigated the expression patterns of Sdf1 and its receptors in avian chimeras after isotopic and isochronic grafting of quail or green-fluorescent–protein-positive (GFP+) transgenic chick neural tubes. NCs were identified using the quail-specific QCPN marker or by their GFP content. When cardiac NC migrated under the pharynx, they progressively lost Cxcr4, and Sdf1 shifted from the ectoderm to the pharyngeal mesoderm (Figure 1F). At E5, cardiac NCs invade the distal part of the outflow tract and, by E8, they reach the ventricular septum (Figure 2A). From the time they invaded the outflow tract until late stages of heart morphogenesis, most cardiac NCs expressed Cxcr7 (Figure 2B–2D; Online Figure II). Interestingly, Sdf1 was found in mesenchymal cells of the conus and truncus closely associated with the NC population (Figure 2B–2D; Online Figure II).

These results show that cardiac NC progression to the heart is associated with SDF1 signaling. Moreover, they identify 2 steps during migration differing in the expression of Cxcr4 and Cxcr7 receptors and in the SDF1 source. First, NCs express Cxcr4 and migrate as a cohort of cells along the ectodermal epithelium, which expresses Sdf1 in a dynamic fashion until they reach the circumpharyngeal region. Second, as cardiac NCs penetrate the outflow tract, they express Cxcr7 instead of Cxcr4 and migrate as a scattered population mingled with an Sdf1-expressing mesenchyme.

**Results**

Loss of Cxcr4 Signaling Leads to Defective Cardiac NC Migration

To assess the role of SDF1 signals in cardiac NC migration, we performed Cxcr4 knockdown experiments. A vector encoding an miRNA directed against Cxcr4 (miRNA-Cxcr4)
was electroporated into the cardiac neural tube of HH9-chick embryos. Efficiency of the miRNA-Cxcr4 to reduce CXCR4 protein level was evidenced in both the electroporated neural tube and cardiac NCs 24 hours postelectroporation (hpe) and could be reversed by cotransfection with a wild-type form of Cxcr4 (Online Figure III). Although initial lateral migration of cardiac NCs was not strongly affected in miRNA-Cxcr4-transfected embryos (Figure 3A–3C), a severe reduction in the number of NCs populating the circumpharyngeal region and migrating into the outflow tract was observed 48 hpe (Figure 3D–3J). Quantitation of the number of GFP-positive NCs revealed a 65% decrease in migrating cardiac NCs after 36 hpe (Figure 3K), which could be attributed essentially to apoptosis, as judged on the detection of activated caspase-3 (Figure 3H, 3J, and 3L). In contrast, NCs following the ventral pathway were not affected by miRNA-Cxcr4 because spinal ganglia formed and enteric NCs succeeded in colonizing the foregut (Online Figure IV).
Because SDF1 signaling is known to regulate both chemotactic migration and cell survival, we overexpressed a construct encoding a dominant-negative form of Cxcr4 (DN-Cxcr4), in which the C-tail of the molecule was deleted. CXCR4 C-tail has been shown to be dispensable for SDF1-dependent survival but critical for chemotaxis, allowing it to discriminate between SDF1 activities during cardiac NC migration. HNK1 immunolabeling revealed that 15 hpe, NCs expressing the DN-Cxcr4 were lagging behind the nonelectroporated NCs (Figure 4A and 4B). Delay in migration was even more pronounced 24 hpe, with most DN-Cxcr4–expressing NCs remaining near the neural tube, whereas their number was decreased by half in the circumpharyngeal region (Figure 4E and 4F). As expected, apoptosis was not increased in DN-Cxcr4–electroporated embryos (not shown), confirming that decrease in NCs in the circumpharyngeal area resulted from reduced or misrouted migration. To trace NC fate later during development, the DN-Cxcr4 construct was coelectroporated with a vector encoding red-fluorescent protein known to be more stable than GFP. Almost no cardiac NCs were observed 72 hpe in the outflow tract of DN-Cxcr4–electroporated embryos, at a time when they normally massively populate this region (Figure 4G–4K). In contrast, DN-Cxcr4–expressing NCs were observed colonizing normally the foregut (Figure 4L). These results indicate that SDF1 signaling drives cardiac NC migration from the neural tube toward the circumpharyngeal region via CXCR4.

Figure 2. Expression profiles of Sdf1 and Cxcr7 during late cardiac neural crest cell (NC) migration in the chick. Green-fluorescent–protein (GFP) visualizations or OCPN immunostainings combined with in situ hybridizations for Sdf1 and Cxcr7. A, Schematic representation of the heart at E5, E6, and E8 showing NC distribution (in red) in the outflow tract at the indicated stage (adapted from Kirby and Hutson). The positions of sections shown in (B–D) are indicated by arrows. B and C, Sections through the outflow tract of E5 (B) and E6 (C) chick-quail chimeras, showing Cxcr7 expression in cardiac NCs (arrows) invading the aorticopulmonary septum. D, Sections through the heart of a GFP+ transgenic chick chimera at E8, showing the persistence of Cxcr7 expression in NC-derived cells of the aorticopulmonary trunk. a indicates aorta; ct, conotruncus; l3-l6, left arch arteries; lv, left ventricle; pt, pulmonary trunk; r3-r6, right arch arteries; and rv, right ventricle. Bars: 100 μm in B, 150 μm in C, and 500 μm in D.
SDF1 Acts as a Chemoattractant for Cardiac NCs

To further determine whether SDF1 could exert a chemotactic effect, we analyzed the migratory response of cardiac NCs confronted to an ectopic source of SDF1. An Sdf1 construct was electroporated into the cardiac neural tube of HH9-chick embryos to induce Sdf1 misexpression close to the NC-emergence site. In these embryos, NCs failed to migrate laterally, and 24 hpe, they formed large cell clusters accumulated near the Sdf1-producing neural tube (Figure 5A and 5D). Interestingly, in high-Sdf1–expressing specimens, NCs originating from the contralateral side were prevented from migrating and accumulated close to the neural tube, showing that excess of secreted SDF1 from one side of the neural tube was sufficient to attract all CXCR4-positive NCs exiting the neural tube. This resulted in the complete absence of NCs in the circumpharyngeal region in both sides of the embryo (Figure 5E and 5F). Forty-eight hpe, NCs were still aggregated near the neural tube in Sdf1-misexpressing embryos, but most of them were apoptotic (Figure 5H and 5I). Sdf1 misexpression in the vagal neural tube produced no apparent effect on ventral NC migration as well as on enteric NC penetration into the foregut (Online Figure IV).

Because Sdf1 exhibits a dynamic spatiotemporal pattern in the ectoderm in relation with NC progression to the circumpharyngeal region, we analyzed the effect of altering this dynamics on cardiac NC migration. Sdf1 was electroporated in the lateral ectoderm to induce its strong, continuous expression from the neural tube up to the circumpharyngeal ridge. Under these conditions, NCs remained accumulated under the Sdf1-expressing ectoderm and failed to reach the circumpharyngeal region (Figure 5G). Thus, these results indicate that SDF1 drives cardiac NC progression under the ectoderm by a chemotactic mechanism requiring precise spatiotemporal regulation of its expression.

Cross-Regulation of SDF1 and CXCR4 Expression During Cardiac NC Migration

To gain insight into the mechanisms controlling spatiotemporal regulation of SDF1 activity during cardiac NC migration,
Figure 4. Expression of a dominant-negative form of CXCR4 impairs cardiac neural crest cell (NC) migration. Green-fluorescent–protein (GFP) or red-fluorescent–protein (RFP) visualizations combined with immunodetections for CXCR4 or HNK1 and 4',6'-diamidino-2-phenylindole (DAPI) stainings. A–D, Sections of control and DN-Cxcr4 embryos 15 (A and B) and 24 (C and D) hours postelectroporation (hpe), showing the delay in migration of DN-Cxcr4–expressing NCs (arrowheads) and their misrouting into the ventral pathway (asterisk). Arrows point at nonexpressing DN-Cxcr4 NCs at the front of migration. E–F, Delineation of the dorsal (D) and circumpharyngeal (CP) quadrants used to quantitate the number of GFP-positive NCs in control and DN-Cxcr4 embryos 24 hpe. G, Schematic representation of an E4.5 embryo showing NC distribution (in red). Arrows indicate the levels of sections shown in J–L. H and I, Lateral views of whole-mounts of control and DN-Cxcr4 embryos 72 hpe, revealing the delayed migration of cardiac NCs toward the heart after DN-Cxcr4 treatment. Asterisk in DN-Cxcr4 and arrow in control point at NCs. J–L, Cross-sections at 2 different levels (indicated in G) of the cardiac region showing the drastic reduction of NCs in the outflow tract (oft) of DN-Cxcr4 embryos (K) but not in the foregut (fg; L). e indicates ectoderm; cnc, cardiac NCs; enc, enteric NCs; h, heart; nt, neural tube; ov, otic vesicle; pb, pulmonary bud; and ph, pharynx. Bars: 100 μm in A and B (left), and C–E; 50 μm in B (right and middle), H, and J; and 200 μm in J–L.
we analyzed the Sdf1 and Cxcr4 expression profiles in the contexts of Cxcr4 loss-of-function and Sdf1 misexpression. In embryos in which Sdf1 was ectopically expressed in the neural tube, CXCR4 was downregulated both in the producing neural tube cells and in cardiac NCs accumulated near the source of Sdf1 (Figure 6A and 6B), indicating that an excessive or prolonged exposure to SDF1 leads to CXCR4 repression. Interestingly, in embryos in which Sdf1 was overexpressed in the neural tube and in DN-Cxcr4–expressing embryos in which NCs failed to populate the pharyngeal arches, a nearly complete disappearance of Sdf1 expression was observed in the lateral ectoderm facing the pharynx (Figure 6C–6E), suggesting that maintenance of Sdf1 expression in the ectoderm depends on cardiac NC–derived signals.

Misregulation of SDF1 Signaling in Cardiac NCs Causes Heart Defects
Finally, we analyzed the consequences of NC migratory defects caused by alterations in SDF1 signaling on heart morphogenesis and the patterning of great arteries. To target all
cardiac NCs, neural tubes were electroporated bilaterally. Hearts were collected and their anatomy and histology examined after completion of cardiac morphogenesis at E9.5. The majority (>80%) of control embryos electroporated with GFP or with a control miRNA exhibited normal hearts (Figure 7A and 7F). In contrast, in embryos electroporated with miRNA-Cxcr4, although the gross anatomy of the heart seemed normal in many cases (not shown), histological analyses revealed large cardiovascular defects, notably VSD in 67% of the embryos and, less frequently, hearts with DORV and transpositions of the great arteries (GAT) (Figure 7B and 7F). Heart defects were even more severe in DN-Cxcr4 embryos, 45% of them presented DORV and transpositions of the great arteries (GAT) (Figure 7B and 7F). This misalignment of the great arteries was systematically accompanied with VSD, which otherwise were observed in 55% of the embryos. Sdf1 misexpression in the neural tube also led to severe heart anomalies. Indeed, 29% of them showed a persistent truncus arteriosus in which the outflow tract was not divided into aorta and pulmonary trunk, giving rise systematically to VSD as a consequence of a missing aorticopulmonary septation (Figure 7D and 7F). VSD were otherwise observed in 43% of the hearts, DORV and transposition of great arteries in 14% each. Therefore, these results show that SDF1 signaling defects in cardiac NCs leads to a large spectrum of cardiac anomalies that phenocopy congenital heart defects, underlining the importance of SDF1 signaling in heart morphogenesis. We also analyzed gut innervation in embryos, in which the SDF1 pathway was altered in cardiac NCs. Immunodetection of Tuj1, a pan-neuronal marker, revealed that, in all miRNA-Cxcr4 and DN-Cxcr4 embryos and in the majority of the Sdf1-misexpressing embryos, gut innervation was normal (Online Figure IV). However, in ≈20% of Sdf1-misexpressing embryos, the hindgut was devoid of enteric innervation, immunolabeling being restricted to the nerve of Remak originating from the sacral neural tube (Online Figure IV). A likely explanation for the occurrence of aganglionic hindgut is that cardiac NC accumulation provoked

Figure 6. Cross-regulation of Sdf1 and Cxcr4 expressions during cardiac neural crest cell (NC) migration. Green-fluorescent-protein (GFP) visualizations combined with immunodetections of Cxcr4 and HNK1 and 4',6'-diamidino-2-phenylindole (DAPI) stainings or with in situ hybridizations for Sdf1. A and B, Cross-sections through the cardiac level of control or Sdf1 embryos 24 hours postelectroporation (hpe), showing repression of Cxcr4 in both NCs (arrows) and neural tube (nt) in Sdf1 embryos. C and D, Whole-mount and cross-sections through 2 different levels of the cardiac region (indicated by double-head arrows) of control (C) and Sdf1 (D) embryos 24 hpe, showing that ectodermal Sdf1 expression (arrowheads) has disappeared on the electroporated side (bracket) in Sdf1 embryos. Arrows indicate NC clusters along the ectopic source of Sdf1. E, A cross-section through the cardiac level of DN-Cxcr4 embryos 24 hpe, showing reduction in ectodermal Sdf1 expression (arrowheads) on the electroporated side (bracket) of Sdf1 embryos. e indicates ectoderm; cnc, cardiac neural crest; h, heart; nt, neural tube; ov, otic vesicle; and ph, pharynx. Bars: 100 μm in all panels.
by Sdf1 misexpression prevented enteric NC migration into the ventral pathway by spatial hindrance, thereby reducing the number of cells colonizing the gut.

Discussion

Chemotactic Guidance of Cardiac NCs to the Heart

Our study demonstrates that cardiac NCs express CXCR4 and are guided during their early migration toward the pharyngeal arches by a chemotactic mechanism elicited by Sdf1 released by the ectoderm. Studies in chick revealed that other chemoattractants may also be implicated in the guidance of cardiac NCs. Fibroblast growth factor (FGF)-8 is expressed in the pharyngeal region and a dominant-negative form of fibroblast growth factor receptor (FGFR)-1 receptor expressed in cardiac NCs provokes a delay in their migration combined with increased cell death. Semaphorin-3C is expressed in the outflow tract, and loss-of-function of its receptor Plexin-D1 results in an NC migration default. Therefore, the ability of cardiac NCs to reach the heart would rely on different chemoattractants operating in a coordinated manner throughout NC navigation. SDF1 and FGF8 signaling would guide cardiac NCs in the lateral path toward the circumpharyngeal region, whereas Semaphorin-3C would drive them to the outflow tract. It will be of interest to test whether SDF1 and FGF8 signaling regulate each other to coordinate initial migration of cardiac NCs.

Sdf1 Drives Cardiac NC Migration and Survival Through the CXCR4 Receptor

Although SDF1 signaling has been shown to regulate both chemotactic migration and cell survival in various cell types, it is not clear which pathways mediate these functions. It has been suggested that CXCR4 is involved in chemotaxis and CXCR7 in survival, but other studies also proposed that both processes may be controlled by CXCR4. In addition, biochemical studies showed that the CXCR4 C-terminal-tail is dispensable for activation of the Jak2/

Figure 7. Misregulation of stromal-derived factor-1 (SDF1) signaling causes cardiac anomalies. Whole-mounts and hematoxylin-eosin–stained longitudinal and transverse sections of hearts from control (A and E), miRNA-Cxcr4 (B), DN-Cxcr4 (C), and Sdf1 (D) embryos at E9.5. Although miRNA-Cxcr4 embryos show ventricular septal defects (VSD; asterisk in B), hearts from DN-Cxcr4 embryos exhibit double-outlet right ventricle (DORV) with a side-by-side position of aorta (a) and pulmonary trunk (pt) both exiting from the right ventricle and resulting in VSD (asterisk in C). Persistent truncus arteriosus (PTA) is observed in hearts from Sdf1 embryos (D), with the incomplete septation of aorta and pulmonary trunk also resulting in VSD (asterisk in D). F. Quantitation of heart defects. a indicates aorta; GAT, great artery transposition; GFP, green-fluorescent protein; la, left atrium; lv, left ventricle; pt, pulmonary trunk; ra, right atrium; and rv, right ventricle. Bars=1 mm.
STAT3 pathway involved in SDF1-dependent survival\textsuperscript{20} or for integrin-mediated adhesion,\textsuperscript{21} but is critical for chemotaxis.\textsuperscript{20} Our results show that CXCR4, but not CXCR7, is expressed in cardiac NCs during their initial migration and that CXCR4 downregulation by \textit{miRNA} leads to their apoptosis, whereas a dominant-negative form of CXCR4 lacking its C-terminal domain induces their misrouting without causing cell death, arguing for a role of CXCR4 in controlling both events in cardiac NCs. Surprisingly, CXCR4 knockdown by \textit{miRNA-Cxcr4} did not prevent initial NC migration, although it was expected to affect both survival and migration. A likely explanation is that because the \textit{miRNA} induced CXCR4 downregulation only after 24 hpe, NCs were capable of initiating migration under the ectoderm but failed to survive as they reached the circumpharyngeal region, that is, precisely at the time when \textit{miRNA-Cxcr4} became fully efficient.

In contrast to \textit{Cxc4}, \textit{Cxc7} was not expressed in cardiac NCs during their initial migration, emphasizing the exclusive role of CXCR4 in mediating the SDF1-guidance cue during this step. However, when NCs invaded the aorticopulmonary trunk, \textit{Cxc4} was replaced by \textit{Cxc7}. In this region, non-NC-derived mesenchymal cells express \textit{Sdf1}, suggesting a role for SDF1 signaling transduced exclusively by CXCR7 at this step. Recent studies showed that CXCR7 may promote cell migration\textsuperscript{26} or proliferation\textsuperscript{27} independently of CXCR4. During heart development, the great arteries derived from cardiac NCs undergo extensive lengthening during septation of the outflow tract.\textsuperscript{8} One possibility is that SDF1 signaling would be implicated in the regulation of cardiac NC proliferation via CXCR7 during this remodeling step.

\textbf{Cross-Regulation of \textit{Sdf1} and \textit{Cxcr4} Expression During Cardiac NC Migration}

An important question concerning chemotaxis is how the ligand is dynamically regulated to create a gradient along the progressing cell population. During germ cell migration in zebrafish, \textit{Sdf1} activity is regulated by CXCR7 expressed in the neighboring somatic cells and acting as SDF1 sequester.\textsuperscript{28} Alternatively, in the lateral line, CXCR7 regulates \textit{Cxc4} transcription in the migrating population to maintain its polarized expression at the front.\textsuperscript{29} Such mechanisms could not account for \textit{Sdf1} regulation in the ectoderm during initial cardiac NC migration as \textit{Cxc7} was not detected. Our results suggest instead an alternative process involving both \textit{Sdf1} and \textit{Cxc4} cross-regulating each other. Indeed, we found that during NC migration, \textit{Sdf1} is expressed initially in the ectoderm up to the neural tube and becomes gradually repressed medially during migration. This suggests that NCs themselves provide the cue for repressing \textit{Sdf1} expression during their progression. However, we also showed that when cardiac NCs fail to reach the pharyngeal arches in \textit{Sdf1} or \textit{DN-Cxcr4}–electroporated embryos, \textit{Sdf1} is downregulated in the ectoderm, suggesting that its expression requires positive signals emanating from NCs. In addition, when \textit{Sdf1} was overexpressed, it induced CXCR4 downregulation. A plausible model accounting for these observations would be that cardiac NCs maintain \textit{Sdf1} expression in the ectoderm during the course of their migration to the pharyngeal arches. After their passage, \textit{Sdf1} would be downregulated in the ectoderm. Conversely, once NCs reach the circumpharyngeal region and pause along the portion of the lateral ectoderm still expressing \textit{Sdf1}, they confront a prolonged SDF1 exposure, causing progressively CXCR4 downregulation on their surface. This would in turn switch SDF1 expression in the ectoderm off, allowing cardiac NC cells to resume migration. A similar regulatory loop has been already proposed for FGF8 effect on cardiac NC.\textsuperscript{22,20} Such molecular cross-talks between cardiac NCs and their environment would then be crucial for their chemotactic guidance toward the heart.

\textbf{SDF1 Role in the Segregation Between Cardiac and Enteric NCs}

Although it has been known for long that the cardiac and enteric NC lineages derive from neural tube regions that overlap in the vagal region at the level of the first 3 somites,\textsuperscript{9,31} mechanisms involved in their segregation have remained elusive. Recent studies showed that cardiac and enteric NCs follow different migration pathways immediately after exiting the neural tube, with first cardiac NCs migrating under the ectoderm and enteric NCs undergoing ventral migration slightly later.\textsuperscript{10} Our results demonstrate for the first time that because of a selective SDF1 chemotactic effect on cardiac NCs, these progenitors are early segregated at onset of migration. In addition, they provide molecular explanation to previous embryology experiments showing that chimera embryos in which the chick cardiac neural tube has been replaced by quail cranial or truncal neural tubes exhibit cardiac anomalies that mirror cardiac NC ablation.\textsuperscript{35} It is likely that cranial and truncal NCs cannot substitute for cardiac NCs because they do not express CXCR4 and, hence, cannot respond to SDF1 chemotaxis. Likewise, when quail neural tubes from somites 1 to 3 are transplanted into the same axial level in older embryos or to somites 5 to 7 level in embryos of the same age, quail NCs are never seen migrating under the ectoderm.\textsuperscript{10} We suggest that NCs coming from the somites 1 to 3 fail to migrate laterally in older embryos because \textit{Sdf1} has already been downregulated in the ectoderm, whereas NCs coming from the somites 5 to 7 do not express CXCR4 and thus cannot interpret the SDF1-guidance cue. However, this model fits with the recent observations that cardiac NCs can only regenerate and repopulate the heart region when ablated before HH9 and not later.\textsuperscript{35} Indeed, at this early stage, \textit{Sdf1} is still expressed in the ectoderm and is, therefore, able to attract the regenerating NCs expressing CXCR4.

\textbf{Misregulation of SDF1 Signaling Leads to Cardiac Anomalies That Phenocopy Cardiac Congenital Pathologies}

\textit{Cxc4} loss-of-function in early migrating cardiac NCs or \textit{Sdf1} misexpression along their migration routes gave rise to a variety of conotruncal malformations. In nearly all cases, VSD, the most frequent heart defect arising after cardiac NC ablation,\textsuperscript{4} was invariably observed. In addition to VSD, DORV and GAT were seen, although less frequently, whereas PTA, considered as the most severe phenotype, was identified in a significant proportion of \textit{Sdf1}-misexpressing embryos. The severity of phenotypes obtained may be related to the strategy used to target SDF1 signaling. Indeed, while \textit{Cxc4} loss-of-function by \textit{miRNA} decreased CXCR4 protein level from
24 hpe, leading to a relatively late NC defect between 24 and 48 hpe, only 15 hours were necessary to reduce NC migration in the DN-Cxcr4–treated embryos. Likewise, the strong effect of Sdf1 misexpression in the neural tube induced an even more rapid and massive accumulation of NCs 6 hpe, resulting in an almost-complete NC depletion from the lateral pathway. Thus, whereas in miRNA-Cxcr4 embryos, a number of NCs could reach the outflow tract and contribute later to septation of the aorto- caval trunk, but not in sufficient amount to form the ventricular septum, in the Sdf1-misexpressing specimens, the nearly complete depletion of cardiac NC impacts also on the formation of the outflow tract. These heart phenotypes mirror those observed in mice defective for Sdf1, Cxcr4, and Cxcr7, which all exhibit VSD14–16 and, occasionally, malalignment of the aorta.34 Although expression of Cxcr4 and Cxcr7 in murine cardiac NCs has not been formally established,35,36 conditional deletions of Cxcr4 in cardiomyocytes36 and of Cxcr7 in endothelial cells34 have not been found to produce VSD, therefore, supporting the idea that heart defects in null mutant mice are related to NC defaults. It will be of interest to analyze the incidence on heart development of conditional Cxcr4 and Cxcr7 deletions in NCs. However, heart phenotypes observed in SDF1-signaling–deficient mice are less severe than in chick. This discrepancy could be attributed to species differences in heart colonize by NCs. Indeed, the use of NC reporters in mice to trace cardiac NCs revealed differences in NC-derived structures in the heart, notably in the aorta and pulmonary trunk, compared with quail-chick tracing.6,37

In humans, mutations in the CXCR4 gene have been found so far associated with warts, hypogammaglobulinemia, infections, and myelokathexis syndrome, a combined immunodeficiency disease.38 Although cardiac anomalies are not commonly detected in this pathology, a recent analysis revealed cardiac defects in some patients with WHIM syndrome.39 The DiGeorge syndrome is a well-described pathology characterized by severe cardiovascular anomalies related to cardiac NC defects.40 Interestingly, an important reduction of Cxcr4 in interneurons has been reported recently in the LgDel mouse model, which phenocopies the 22q11.2 deletion of the DiGeorge syndrome, possibly accounting for the cortical circuit disorders observed in this syndrome.41 This raises the intriguing possibility that cardiac anomalies of the DiGeorge syndrome might be related at least partly to diminished Cxcr4 expression.

Taken together, our results demonstrate that disrupting SDF1 signaling in cardiac NCs impairs their migration massively, thus leading to heart anomalies, and opens perspectives for identifying new genes in cardiac congenital pathologies in humans.

Acknowledgments
We thank S. Gourret for illustration, our colleagues for constructs, probes, and antibodies, and the GFP+transgenic-egg resource at the Roslin Institute.

Sources of Funding
This work was supported by the CNRS, the Université Pierre et Marie Curie, and the Association Française contre les Myopathies (Grant No. 11405).

Disclosures
Sophie Escot was a recipient of a doctoral fellowship from the Ministère de l’Enseignement Supérieur et de la Recherche.

References


---

**Novelty and Significance**

**What Is Known?**

- During embryogenesis, cardiac neural crest cells (NC) migrate from the future spinal cord to give rise to the smooth muscle of the cardiac outflow tract and the great vessels, as well as the septum that separate ventricles.
- Some of the signaling pathways that regulate cardiac NC migration have been identified.
- Abnormalities in cardiac NC migration have been implicated in congenital heart diseases in humans.

**What New Information Does This Article Contribute?**

- In the chick embryo, expression of the chemokine, stromal-derived factor-1 (SDF1), is tightly coordinated with the progression of cardiac NCs expressing its CXCR4 receptor during their early step of migration toward the heart.
- SDF1 signaling selectively drives cardiac NC migration toward the heart via a chemotactic mechanism.
- Misregulation of SDF1 signaling results in severe cardiovascular defects, identifying SDF1 and its receptor CXCR4 as candidate genes responsible for cardiac congenital pathologies in human.

Cardiac NCs contribute to heart morphogenesis by giving rise to smooth muscle of the outflow tract and great vessels, as well as part of the ventricular septum. Failure in cardiac NC development results in outflow tract and ventricular septation defects commonly observed in congenital heart diseases. However, the molecular mechanisms driving cardiac NC migration toward the heart have not been completely elucidated. We show that in the chick embryo, the SDF1 signaling pathway plays an essential role in cardiac NC migration toward the heart. Loss-of-function of CXCR4 (the SDF1 receptor) causes delayed migration and enhanced cell death of cardiac NCs, whereas misexpression of SDF1 provokes cardiac NC diversion from their normal migration pathway, indicating that SDF1 acts as a chemoattractant for cardiac NCs. These findings suggest that SDF1 and its receptor CXCR4 may be candidate genes responsible for cardiac congenital pathologies in humans.
Misregulation of SDF1-CXCR4 Signaling Impairs Early Cardiac Neural Crest Cell Migration Leading to Conotruncal Defects
Sophie Escot, Cédrine Blavet, Sonja Härtle, Jean-Loup Duband and Claire Fournier-Thibault

_Circ Res._ 2013;113:505-516; originally published online July 9, 2013; doi: 10.1161/CIRCRESAHA.113.301333
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/5/505

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/07/09/CIRCRESAHA.113.301333.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
ONLINE SUPPLEMENTARY DATA

DETAILED METHODS

Embryos
Fertilized, normal chick and quail eggs were from commercial sources and green fluorescent protein positive (GFP+) transgenic chicken eggs\(^1\) from the Roslin Institute (University of Edinburgh, UK). Eggs were incubated at 38°C in a humidified incubator until appropriate stages. Embryos were staged according to the Hamburger and Hamilton (HH) chart \(^2\) or referred to embryonic day of development (E).

Embryonic chimeras
Grafting experiments were performed using classical procedures described previously \(^3\). Briefly, cardiac-level neural tubes from pinned-out quail embryo or GFP+ transgenic chick embryo donors at HH9 (E1.5) were excised using sharpened microscalpels after brief dispase II (Roche) enzymatic treatment and transplanted isotopically and isochronically into chick hosts in ovo, in which their equivalents have been removed surgically. After sealing with tape, the eggs were re-incubated for up to 8 days before analysis.

In ovo electroporation
For transient transgenesis in chick, the following expression plasmids were used: pCA\(\beta\) containing GFP (0.4 g/ml), pCAAGS containing RFP (0.4 g/ml), pCA\(\beta\) containing full-length chick Sdf1 (0.8 g/ml) subcloned from chicken EST clones (MRC chicken database), and pCA\(\beta\) containing a chick dominant-negative form of CXCR4 (\(DN\text{-}Cxc\text{r}4\), 0.8 g/ml). The \(DN\text{-}Cxc\text{r}4\) was constructed by mutating the amino acid 217 to introduce a stop codon. This results in the truncation of the intracytoplasmic tail of CXCR4 protein, thereby eliminating the sequences that confer G protein phosphorylation activity and \(\beta\)-arrestin recruitment, the major signal transduction pathways implicated
in its chemotactic activity\(^4\). For miRNA experiments, pcDNA6.1 Gw/EmGFP-miR chick Cxcr4 (miRNA-Cxcr4, 1.0 g/ml) and pcDNA6.1 Gw/EmGFP-miR control (miRNA-cont, 1.0 g/ml) were constructed as described in the manufacturer\'s instructions using BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen, K4935-00). The three following CXCR4 target sequences were used: TGTTGGCTGCCGTATTACATT,\(^5\) AAGCTGTTGGCTGAGAAGATT and GATTGGCTCAGCTGACTAT.\(^6\) All three miRNAs tested individually were found to give comparable results.

For in ovo neural tube and ectoderm electroporation, plasmids of interest were mixed with plasmids encoding GFP or RFP at the concentrations indicated above and microinjected into the lumen of the neural tube or over the ectoderm of HH9 embryos. Electrodes (CUY610 platinum-coated, Sonidel, Ireland) were applied onto the vitelline membrane on either side of the appropriate neural tube region and 5 square pulses (20 V, 50 ms length, 500 ms gap) were delivered. In some experiments, pulses were delivered bilaterally to electroporate both sides of the neural tube. Eggs were reincubated for up to 8 days after electroporation, embryos harvested in phosphate-buffered saline (PBS) and monitored for GFP or RFP fluorescence at the appropriate axial levels. The efficiency of the transfections was verified by in situ hybridisation on whole mount or on sections for Sdf1 and by immunofluorescent labelling on whole mount or on sections for CXCR4.

**Immunostaining, in situ hybridisation, and histological analyses**

Once harvested in PBS and monitored for GFP or RFP expression, embryos were treated for in situ hybridisation or immunostaining on whole embryos or sections. Embryos were fixed in 4% formaldehyde with 2 mM EGTA and progressively dehydrated in methanol. Hybridizations were performed on rehydrated embryos as described previously.\(^7\) For immunostainings on whole embryos, embryos were permeabilized with Triton X100. For hybridisations and immunostaining on sections, embryos were fixed in a 4% paraformaldehyde solution in PBS supplemented with 4% sucrose and 0.1 mM CaCl\(_2\), rinsed in PBS, embedded in a 15% sucrose solution, frozen in chilled isopentane before cryostat sectioning at 10-20 \(\mu\)m. Sections were collected on Superfrost/Plus slides (CML, France) and processed for immunolabelling or in situ hybridisation as described previously.\(^7\) For in
situ hybridisations, the following riboprobes were used: Sox10 (from P. Scotting), and Sdf-1, Cxcr4, Cxcr7 which were produced from EST clones (respectively from Medical Research Council, Delaware Biotechnology Institute, and ARK Genomics). For immunofluorescent labellings, the following primary antibodies were used: anti-phospho-histone-3 (Upstate Biotechnology, 1:1000), anti-cleaved caspase-3 (Cell Signalling, 1:100), anti-NC1/HNK18 (1:10), anti-QCPN (Developmental Study Hybridoma Bank at Iowa University, pure supernatant), anti-TuJ1 (Chemicon, 1:200), anti-GFP (Roche, 1:500) and anti-chicken CXCR4 (1:1000). To generate the anti-CXCR4 antibody, mice were immunized with HEK293T cells transfected with a chicken CXCR4-GFP construct. Murine spleen cells were fused to SP2/0 cells and supernatants of resulting hybridomas were tested by flow cytometry on transfected and untransfected HEK293T cells (Supplementary Figure 9). For QCPN and CXCR4 stainings, sections were permeabilized with Triton X100. Immunolabellings were performed using appropriate secondary antibodies conjugated to Alexa Fluor 488 or 555 (Invitrogen). Nuclei were visualised with DAPI (Sigma). For classical histological analyses, dissected hearts were fixed, dehydrated in a graded series of ethanol, embedded in paraffin, sectionned at 8 μm and stained with hematoxylin/eosin using standard procedures. Preparations were observed with a Nikon microscope equipped for epifluorescence and images were collected using the QCapture Pro software (QImaging) and processed using Adobe Photoshop software.

Quantitative analyses

Numbers of migrating neural crest cells (NCs) per section were quantitated in miRNA-Cxcr4-electroporated embryos by counting the number of GFP+ cells present at the periphery of the neural tube on every 10 μm-thick section throughout the electroporated region, representing about 20 to 30 sections per embryo. Likewise, quantitation of apoptosis was performed by counting the number of activated caspase-3-positive cells on each section. The number of migrating NCs in DN-Cxcr4-electroporated embryos was quantituated by defining dorsal and circumpharyngeal quadrants on sections of electroporated embryos: the dorsal part was defined as the part of the section facing the dorsal two-thirds of the neural tube, the circumpharyngeal part was defined as the region comprised between the ventral third of the neural tube and the circumpharyngeal region. GFP-positive NCs were
counted in each area on every 10 μm-thick section of the electroporated region. Statistical significances were evaluated using the Student’s T test.

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure I: Expression profiles of Sdf1, Cxcr4 and Cxcr7 during chick embryonic development. In situ hybridisations for Sdf1, Cxcr4 and Cxcr7 and immunolabellings for HNK1. (A-F’) Whole-mount of HH9 (A,A’), HH10 (B,B’), HH11 (C,C’), HH13 (D,D’), HH21 (E,E’) and HH23 (F,F’) embryos showing that although Sdf1 and its receptor are expressed in a large variety of embryonic tissues, they are prominent at the cardiac level (indicated by brackets) from HH10 to HH21. (G, H) Serial cross-sections of HH12 (G) and HH14 (H) embryos, showing the absence of Cxcr7 expression in cardiac NCs (arrows) during their migration to the pharynx. (I) Serial sections of a HH14 embryo, illustrating the absence of both Cxcr4 and Cxcr7 in enteric NCs (enc) migrating in the ventral pathway and entering the foregut (fg). a: aorta, h: heart, nt: neural tube, ph: pharynx. Bars: 150 μm in A-E’; 50 μm in F,F’; 150 μm in G-I.

Supplementary Figure II: Expression profiles of Sdf1 and Cxcr7 during formation of the NC-derived aorta and pulmonary trunk in the chick. GFP visualisations and QCPN immunostainings combined with in situ hybridisations for Sdf1 and Cxcr7 and DAPI stainings. (A,D) Schematic representation of NC (in red) migration during heart development (adapted from Ref. 9). (B,C) Detail views of sections (level indicated in A) through the aortico-pulmonary septum of E6 chick-quail chimeras, showing Cxcr7 expression in most QCPN-positive cardiac NCs invading the Sdf1-positive mesenchyme of the proximal conotruncus. (E,F) Detail views of sections through the aorta (level indicated in D) of a GFP-positive chick chimera at E8, showing persistence of Cxcr7 expression in the vast majority of NC-derived cells along the aorta. a: aorta, l3-l6: left arch arteries, lv: left ventricle, r3-r6: right arch arteries, rv: right ventricle, pt: pulmonary trunk. Bars: 50μm in A-B; 150 μm in C-D.
**Supplementary Figure III:** CXCR4 loss-of-function by *miRNA* leads to specific down-regulation of CXCR4. GFP visualisations combined with immunodetections for HNK1 or CXCR4 and DAPI stainings. (A) Whole-mount and cross-sections of *miRNA-Cxcr4*-electroporated embryos 24 hpe, showing CXCR4 down-regulation in both neural tube (nt) and NCs (arrows) on the electroporated side compared to the control side. Arrowheads point at non-electroporated NCs at the front of migration expressing CXCR4. (B) Cross-sections of *miRNA-control*-electroporated embryos 24 hpe, showing CXCR4 expression in both the neural tube and the migrating NCs on the electroporated side. Bottom panels are detail views of the circumpharyngeal region delineated by insets in upper panels. (C, D) Cross-sections of *miRNA-Cxcr4*-electroporated (C) and control (D) embryos 48 hpe, showing that CXCR4 down-regulation persists in the neural tube (arrows), illustrating the long-term effect of the *miRNA-Cxcr4*. Note that at this stage of development, CXCR4 expression is no longer expressed in NCs even in non-electroporated cells (compare with Fig. 1) and that it is only found in endothelial cells (arrowheads). In the control embryo, NCs (arrows) migrate normally on the electroporated side. (E) Cross-sections of 24 hpe embryos co-electroporated with *miRNA-Cxcr4* and full-length Cxcr4 constructs, showing the normal migration of NCs (arrows) when *miRNA-Cxcr4* effect is rescued by coexpression with Cxcr4. The right panel is a detail view (inset) of the circumpharyngeal region showing a merge picture of GFP and CXCR4 stainings. e : ectoderm, nt : neural tube, ov : otic vesicle, ph : pharynx. Bars: 50 μm in AA’; 100 μm in B-E’.

**Supplementary Figure IV:** Effect of SDF1 misregulation on other NC populations derived from the vagal neural tube. (A, B) Visualisation of GFP combined with HNK1 immunolabelling on cross-sections through the vagal region of a *miRNA-Cxcr4*-electroporated embryo (A) 48 hpe and an *Sdf1*-electroporated embryo (B) 24 hpe, revealing that NCs destined to form the spinal or dorsal root ganglia (drg) normally migrate in both cases. (C-F) Left panels: visualisation of GFP combined with HNK1 immunolabelling on cross-sections at the level of the foregut (fg) in control (C), *miRNA-Cxcr4*- (D), *DN-Cxcr4* - (E), and *Sdf1*-electroporated (F) embryos 48 hpe, showing normal colonisation of the foregut by enteric NCs (enc) under all experimental conditions. Right panels: immunodetection of Tuj1 on whole mounts of hindguts from control (C), *miRNA-Cxcr4*- (D), *DN-Cxcr4* - (E), and *Sdf1*-
electroporated (F) embryos 8 days after electroporation, showing that the hindgut has been normally colonised by enteric NCs, except in some Sdf1-electroporated specimens in which the hindgut is devoid of enteric innervation. Note that the node of Remak (NoR) deriving from truncal NCs has not been affected. Bars: 100 μm.

**Supplementary Figure V:** Assessment of the specificity of the CXCR4 antibody A mixture of untransfected HEK293T cells and CXCR4-GFP-transfected HEK293T cells was stained with the anti-chicken CXCR4 (clone 9D9) followed by goat-anti-mouse-IgG2a conjugated to phycoerythrin and analysed by flow cytometry, demonstrating that only GFP-positive, CXCR4-expressing cells are stained.

**SUPPLEMENTAL REFERENCES**
