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Role of CXCR4/SDF1 signaling in the initial migration of cardiac neural crest cells

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ABSTRACT

Neural crest cells are migratory and multipotent cell populations that give rise to various derivatives during the development of vertebrate embryos. The cells are specified in the neural folds and undergo epithelial mesenchymal transition (EMT) to delaminate and then migrate within the embryo. Cardiac neural crest cells originate from the caudal hindbrain and migrate through the pharyngeal arches to the heart. It has been shown that CXCR4/SDF1 signaling control cardiac neural crest cells migration toward pharyngeal arches in chicken embryos. Here, we investigated the effect of disruption of CXCR4/SDF1 signaling on cardiac neural crest cell migration by implanting beads into the lumen of the closing neural tube. We first observed that a CXCR4 antagonist inhibited initial cardiac neural crest migration. We also found that an ectopic source of SDF1 caused the accumulation of cardiac neural crest cells in the dorsal neural tube and the invasion of cardiac neural crest cells into the neural tube. Furthermore, disruption of signaling led to disorganization of the neural tube basement membrane but did not affect neural crest EMT. Overall, our data indicate that CXCR4/SDF1 signaling is critical for as early as the onset of cardiac neural crest cell migration after the delamination.

1. Introduction

Neural crest cells are a multipotent cell population unique to vertebrates and give rise to various derivatives. The cells are specified in the neural folds during embryonic neurulation. Then, the premigratory neural crest undergoes epithelial mesenchymal transition (EMT) to delaminate, and delaminated neural crest cells migrate widely within developing embryos. The production of neural crest cells from the neural tube is a transient process that is controlled by a gene regulatory cascade orchestrated by various molecules [1]. A remarkable gene known as the neural crest marker *Sox10* is expressed in premigratory neural crest cells, and its expression is maintained during migration [2]. *Snail1/2* are well known crucial EMT regulators [3]. During EMT, Snail2 plays roles in cadherin switching, directly repressing *Cadherin 6B* (*Cad6B*), which is expressed in premigratory neural crest cells, and regulating basement membrane remodeling [4–7].

Cardiac neural crest cells are a subpopulation of the neural crest that originate from the caudal hindbrain [8]. In avian embryos, this cell population migrates through pharyngeal arches and contributes to cardiovascular formation, including the aorticopulmonary septum, valves and cardiac ganglia [9–11]. The phenotypes resulting from surgical ablation of cardiac neural crest in chickens or genetic ablation of the cardiac neural crest cells in mice mirror congenital heart diseases in humans [12–15].

Since deficient mice of the C-X-C chemokine receptor *CXCR4* or its ligand *SDF1* (*CXCL12*) exhibit heart defects, especially lack of the septum, it has been suggested that CXCR4/SDF1 signaling may contribute to guiding cardiac neural crest cells into the heart [16,17]. Recently, it was reported that *CXCR4* is expressed in migrating cardiac neural crest cells, while *SDF1* is expressed in ectodermal cells and that CXCR4/SDF1 signaling regulates the “early migration” of cardiac neural crest cells to pharyngeal arches [18]. Dysregulation of CXCR4/SDF1 signaling leads to a heart defect due to abnormal cardiac neural crest migration. *CXCR4* knockdown or dominant-negative CXCR4 expression leads to reduced or misrouted migration, although it does not affect the “initial migration” of cardiac neural crest cells, probably due to the limitations of the transfection experiment [18]. Thus, CXCR4/SDF1 signaling is clearly required for the appropriate early migration of neural crest cells toward the pharyngeal arches, whereas it remains unclear whether CXCR4/SDF1 plays crucial roles in the initial migration after delamination.

In this study, we developed a method using bead implantation as an

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effective way to investigate the initial migration step of cardiac neural crest cells from the neural tube of chicken embryos. To disrupt endogenous CXCR4/SDF1 signaling for cardiac neural crest cell migration, we implanted beads soaked with the CXCR4 antagonist AMD3100 or with SDF1 protein into the closing neural tube at the axial level of the cardiac neural crest and then examined the expression of neural crest markers in the embryos. These results showed that (i) the inhibitor in the lumen of the neural tube caused the accumulation of cardiac neural crest cells in the dorsal neural tube by inhibiting the initial cardiac neural crest cell migration and that (ii) the ectopic ligand led to the accumulation of cardiac neural crest cells in the dorsal neural tube or mismigration into the neural tube. Furthermore, we verified the processes of EMT and delamination of the neural crest and the formation of a basement membrane in the neural tube of bead-implanted embryos. We found that the accumulated cell population interferes with the basement membrane of the dorsal neural tube. In contrast, the disruption of signaling did not affect the neural crest EMT process. Thus, we propose that CXCR4-mediated regulation is critical even for the initial migration following EMT.

2. Materials and methods

2.1. Chicken embryos

Fertilized chicken eggs were purchased from Shimojima and Inoue farms (Kanagawa, Japan) and incubated at 38 °C to obtain the desired embryonic stages (HH) [19]. All experiments were conducted following the guidelines of the Animal Care Committee of Kobe University.

2.2. Molecular cloning and plasmids

Chicken *CXCR4*, *SDF1 or RhoB* fragments were amplified from the cDNA of HH12 chicken embryos as described previously by Tani-Matsuhana et al. [20], and inserted into the *Xho*I/*Eco*RI sites of the pBluescript vector. The following primers were used:

CXCR4 Fw, 5′-ccgctcgagACGGTTTGGATCTGTCCTCTGG-3′,
CXCR4 Rv, 5′-ccggaattcTCTTCAAGGCTTTGCGCTTCTG -3’
SDF1 Fw, 5′-ccgctcgagTTTGCCCTGGCAGTCATCTC-3′,
SDF1 Rv, 5′-ccggaattcACATGGTTAAAGCCAGCCCT-3’.
RhoB Fw1, 5′-CCATCCGCAAGAAGCTGGTG -3′,
RhoB Rv1, 5′-ATAGGACCTTGCAGCAATTG-3′,
RhoB Fw2, 5′-gggctcgagGTGGCAAGACCTGCCTCCTC-3′,
RhoB Rv2, 5′-ggggaattcCAGCCGTTCTGAGTGCCGTA-3’. RhoB fragment was amplified by nested-PCR with 1st primer set of RhoB Fw1/Rv1 and 2nd primer sets of RhoB Fw2/Rv2. Restriction enzyme recognition sequences and three additional nucleotides are shown in lower case.

2.3. Ex ovo electroporation

Reporter plasmids were electroporated ex ovo into the ectodermal layer of stage HH4 embryos. The protocol was performed as described previously [21]. *FoxD3* NC2-EGFP [22] was used at a concentration of 1 μg/mL.

2.4. Bead implantation

Agarose beads (Bio-Rad, Affi-Gel Blue Gel 1537302) were washed three times with PBS and then soaked in PBS, 1 μg/ml SDF1 solution (Petro Tech AF-300-28 A) or 15 mg/ml AMD3100 solution (Sigma–Aldrich, A5602) overnight at 4 °C. The beads were implanted into the closing neural tube at the axial level of the cardiac neural crest in wild-type embryos or reporter-electroporated embryos after a slit at the dorsal closing site of neural tube was made with a tungsten needle. The implanted embryos were developed on agar culture media until HH13.

2.5. In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed as previously described [23]. For *CXCR4*, *SDF1 or RhoB* probe synthesis, the plasmid was linearized with *Xho*I and transcribed with T3 RNA polymerase. The plasmid for the *Sox10* probe [2] was linearized with *Hind*III and transcribed with T3 RNA polymerase.

Whole-mount immunohistochemistry for CXCR4, laminin and GFP was performed using previously described procedures [21]. For Snail2 and Cad6B, chicken embryos were fixed with 4 % paraformaldehyde (PFA) for 15 min at room temperature and washed in TBST + Ca^{2+}. Then, the embryos were blocked with 10 % donkey serum in TBST + Ca^{2+} for 1 h, and the primary antibodies diluted in blocking solution were added and incubated overnight at 4 °C. The embryos were then washed with TBST + Ca^{2+}, incubated with secondary antibodies diluted with blocking solution for 3 h at room temperature, and then washed with TBST + Ca^{2+}. The following antibodies were used: mouse anti-CXCR4 (Bio-Rad, MCA6012GA), goat anti-GFP (abcam, ab6673), rabbit anti-Slug/Snail2 (Cell Signaling, 9585), rabbit anti-Laminin (Sigma–Aldrich, L9393), mouse anti-Cadherin6B (DSHB, CCD6B-1), donkey anti-mouse IgG conjugated with Alexa Fluor 488, anti-goat IgG conjugated with Alexa Fluor 488, and anti-rabbit IgG conjugated with Alexa Fluor 488 or 594 (Invitrogen, A21202, A11055, A21207, A11034). DAPI was used for nuclear counterstaining.

Cryosectioning of the embryo was performed as described previously [24].

2.6. Imaging

Whole embryos were imaged using SMZ1500 and AZ100 M microscopes (Nikon) and a Ds-Ri1 camera (Nikon). For transverse sections, an ECLIPSE Ni microscope (Nikon) and a Ds-Ri2 camera (Nikon) were used for image acquisition.

3. Results and discussion

3.1. The expression patterns of CXCR4 and SDF1 in the cardiac neural crest region during chicken embryonic development

To confirm the expression patterns of *CXCR4* and *SDF1* at the embryonic stages of cardiac neural crest migration, we performed whole-mount in situ hybridization against *CXCR4* and *SDF1* in chicken embryos. At stage HH10, *CXCR4* was weakly expressed in rhombomeres 5 and 6, which are sources of cardiac neural crest cells (Fig. S1A), and the expression signal was detected in migrating cardiac neural crest cells at stages of HH11-13 (Fig. S1B-D and J). We confirmed that migrating cardiac neural crest cells by immunostaining with an anti-CXCR4 antibody (Fig. S1I). *SDF1* expression was detected at the ectoderm at the axial level of the cardiac neural crest at stage HH10 (Fig. S1E), and the expressed region shifted to the ventral side of the embryos (Fig. S1F-H and K). These findings are consistent with the previous reports [18,25].

3.2. SDF1 and AMD3100 beads affect cardiac neural crest cell migration

We next investigated whether disruption of SDF1/CXCR4 signaling by bead implantation affects the migration of cardiac neural crest cells, especially initial migration, in chicken embryos. To this end, we implanted SDF1- or AMD3100 (CXCR4 antagonist)-soaked beads into the lumen of the neural tube at the axial level of the cardiac neural crest at stage $HH10^{+}$, when the neural tube is just closing. Then, the implanted embryos were cultured to reach the cardiac neural crest migration stage, HH13. In the control embryos implanted with PBS beads, *Sox10* expression was observed in migrating cardiac neural crest cells as bilateral streams posterior to the otic vesicle (Fig. 1A). In contrast, the cardiac neural crest cell stream was reduced in SDF1 bead-implanted embryos, while the streams was not observed in AMD3100

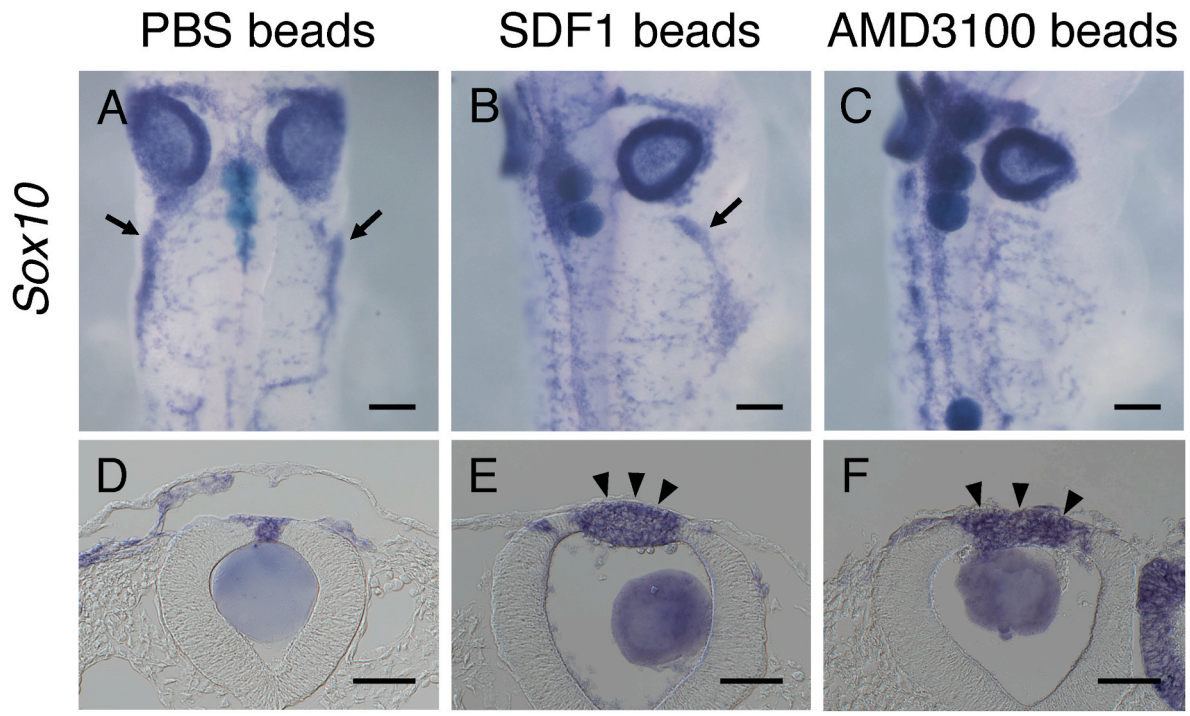

Fig. 1. Ectopic SDF1 and a CXCR4 antagonist (AMD3100) affect cardiac neural crest cell migration. Expression patterns of *Sox10* in embryos implanted with PBS (A), SDF1 (B) or AMD3100 beads(C). The embryos were sectioned at the post otic level of the implanted beads (D–F). The arrows and arrowheads show migrating cardiac neural crest cells and the expanded region for *Sox10* expression, respectively. N = 3 (A), 2 (B), 2 (C). Scale bars: 100 μm (A–C) or 50 μm (D–F).

bead-implanted embryos (Fig. 1B and C). In transverse sections at the level of post otic vesicle, we observed that *Sox10*-expressing cell population was expanded in the dorsal neural tube in the presence of SDF1 or AMD3100 beads (Fig. 1D–F). These findings strongly suggest that disruption of SDF1/CXCR4 signaling results in the accumulation of migrating cardiac neural crest cells in the dorsal neural tube. However, the possibility remains that delamination and/or specification of cardiac neural crest cells may be affected.

3.3. Migrating cardiac neural crest cells form a cluster on/in the dorsal neural tube in response to SDF1 or AMD3100 beads

To determine whether the implantation of SDF1 or AMD3100 beads affects neural crest specification or the migration of cardiac neural crest cells, we labeled migrating neural crest cells with EGFP in embryos. For this purpose, we first electroporated HH4 embryos with the *FoxD3* NC2-EGFP reporter, which is active only in migrating cardiac and trunk neural crest cells and not in premigratory neural crest cells (Fig. 2A) [22]. After the electroporated embryos were cultured until stage HH10^{+}, we implanted SDF1 or AMD3100 beads into the neural tube at the level of the cardiac neural crest and cultured them again until stage HH13 (Fig. 2A). As a result, in the embryos treated with PBS beads, most of the GFP-positive cardiac neural crest cells migrated normally as the bilateral streams (Fig. 2B). The embryos were subsequently sectioned at the axial level of the post otic vesicle. The transverse section revealed that a large population of GFP-positive cardiac neural crest cells migrated to the ventral side, while some of them were just delaminated from the neural tube (Fig. 2E–H, K). In the embryos with SDF1 beads, however, GFP-positive neural crest cells were observed predominantly in the dorsal neural tube and the cardiac neural crest streams almost disappeared (Fig. 2C). When we observed the sections in detail, the GFP-positive cardiac neural crest cells populated the dorsal neuroepithelium, and some of the cells invaded the lumen of the neural tube in the embryos with SDF1 beads (Fig. 2F–I, L). In embryos with AMD3100 beads, GFP-positive cardiac neural crest cells tended to populate the dorsal neural tube, and the cardiac neural crest cells migrated to the ventral side was reduced as compared to the embryos implanted with PBS beads (Fig. 2D). The sections revealed that the GFP-positive cells lay between the nonneural ectoderm and neural tube (Fig. 2G–J, M). These findings showed that the initial migration of cardiac neural crest cells is inhibited by the disruption of CXCR4/SDF1 signaling, resulting in the accumulation of migrating cells in or near the dorsal neural tube. However, the accumulation of migrating cells labeled with the *FoxD3* reporter seemed to be smaller than the *Sox10*-expressing cell population expanded in the dorsal neural tube. This result led us to hypothesize that disruption of CXCR4/SDF1 signaling also impairs processes such as EMT and delamination, which precede the initial migration of cardiac neural crest cells.

3.4. Disruption of CXCR4/SDF1 signaling leads to disorganization of the basement membrane of the dorsal neural tube

EMT and delamination in the neural tube are associated with remodeling of the basement membrane. To determine whether the organization of neural tube basement membranes is affected by disruption of CXCR4/SDF1 signaling, we examined the expression of laminin, a marker for the basement membrane, in PBS-, SDF1- or AMD3100 bead-implanted embryos (Fig. 3). The embryos were immunostained with antibodies against laminin and CXCR4 at stage HH13 and transverse sectioned at the cardiac neural crest axial level. In PBS bead-implanted embryos, cardiac neural crest cells expressing CXCR4 migrated toward the ventral side, and only a small population remained within the dorsal neural tube (Fig. 3A–G, J). Laminin was localized in both the nonneural ectoderm and the closed neural tube (Fig. 3D–G, J). In contrast, the sections of embryos with SDF1 or AMD3100 beads showed accumulated CXCR4-positive cardiac neural crest cells in the dorsal neural tube and reduced laminin expression in this population (Fig. 3B, C, E, F, H, I, K L). In the nonneural ectoderm, laminin expression did not change in any bead-implanted embryos. Thus, we considered these phenotypes of disorganization of the basement membrane of the neural tube to be due to the accumulation of cardiac neural crest cells.

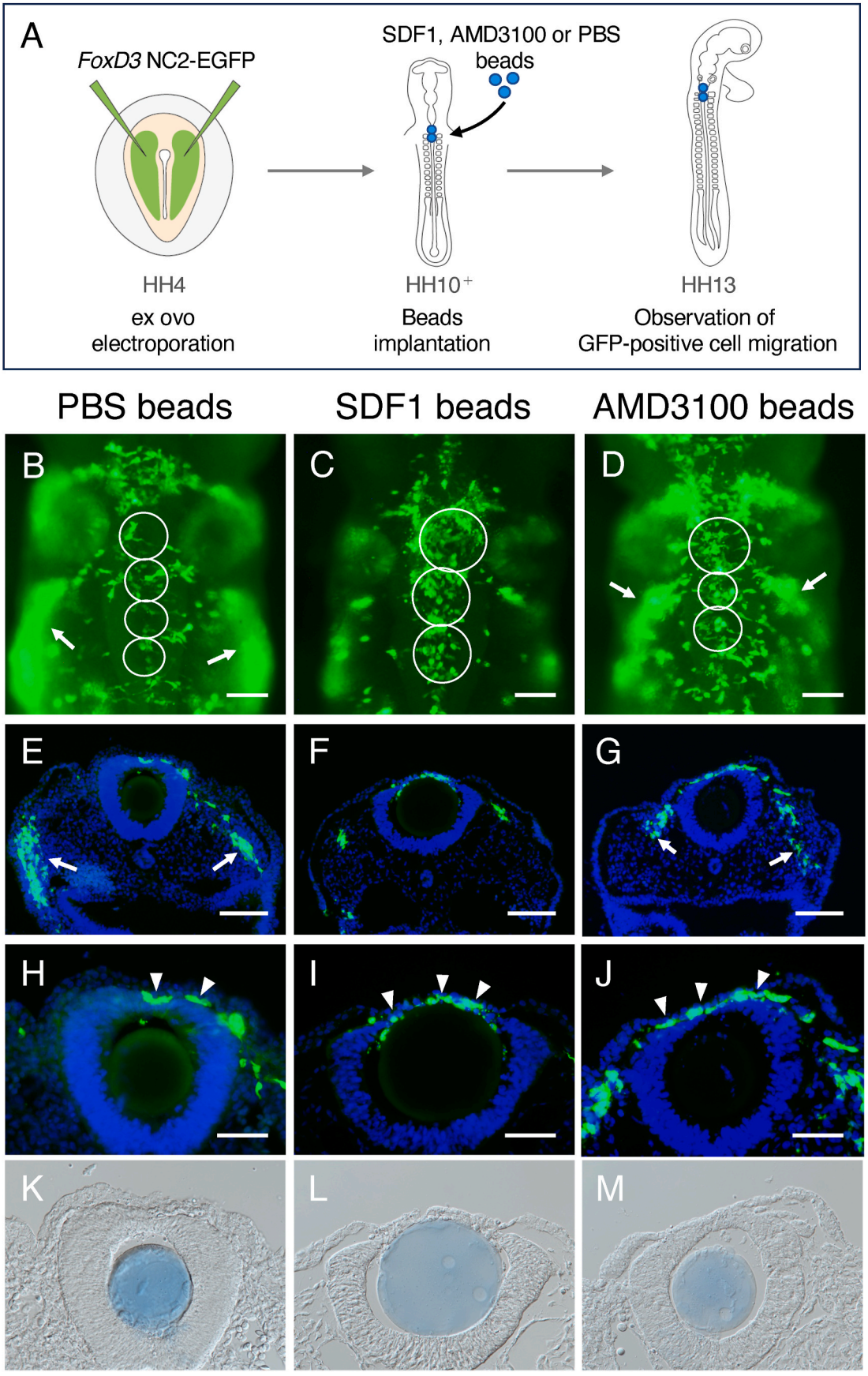

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Fig. 2. Migrating cardiac neural crest cells are populated on/in dorsal neural tubes in embryos implanted with SDF1 or AMD3100 beads. (A) Diagram of the combination of ex ovo electroporation and implantation of beads. The *FoxD3* NC2-EGFP reporter plasmid was electroporated into both sides of the embryos. (B–D) Embryos were implanted with PBS-, SDF- or AMD3100-soaked beads at stage HH10^{+}. In these embryos, GFP reporter expression was detected with an anti-GFP antibody by whole-mount immunostaining at stage HH13. (E–M) Embryos were sectioned at the level of the otic vesicle and implanted beads. (H–J) Enlarged views of the dorsal neural tube region. (K–M) Images in the bright field of Panels H–J. The arrows, arrowheads and white circles show the migrating cardiac neural crest streams, the populated/accumulated migrating cardiac neural crest cells in/on the dorsal neural tube and the position of the implanted beads, respectively. All the nuclei were stained with DAPI. N = 4 (B), 4 (C), 3 (D). Scale bars: 100 μm (B–G) or 50 μm (H–J).

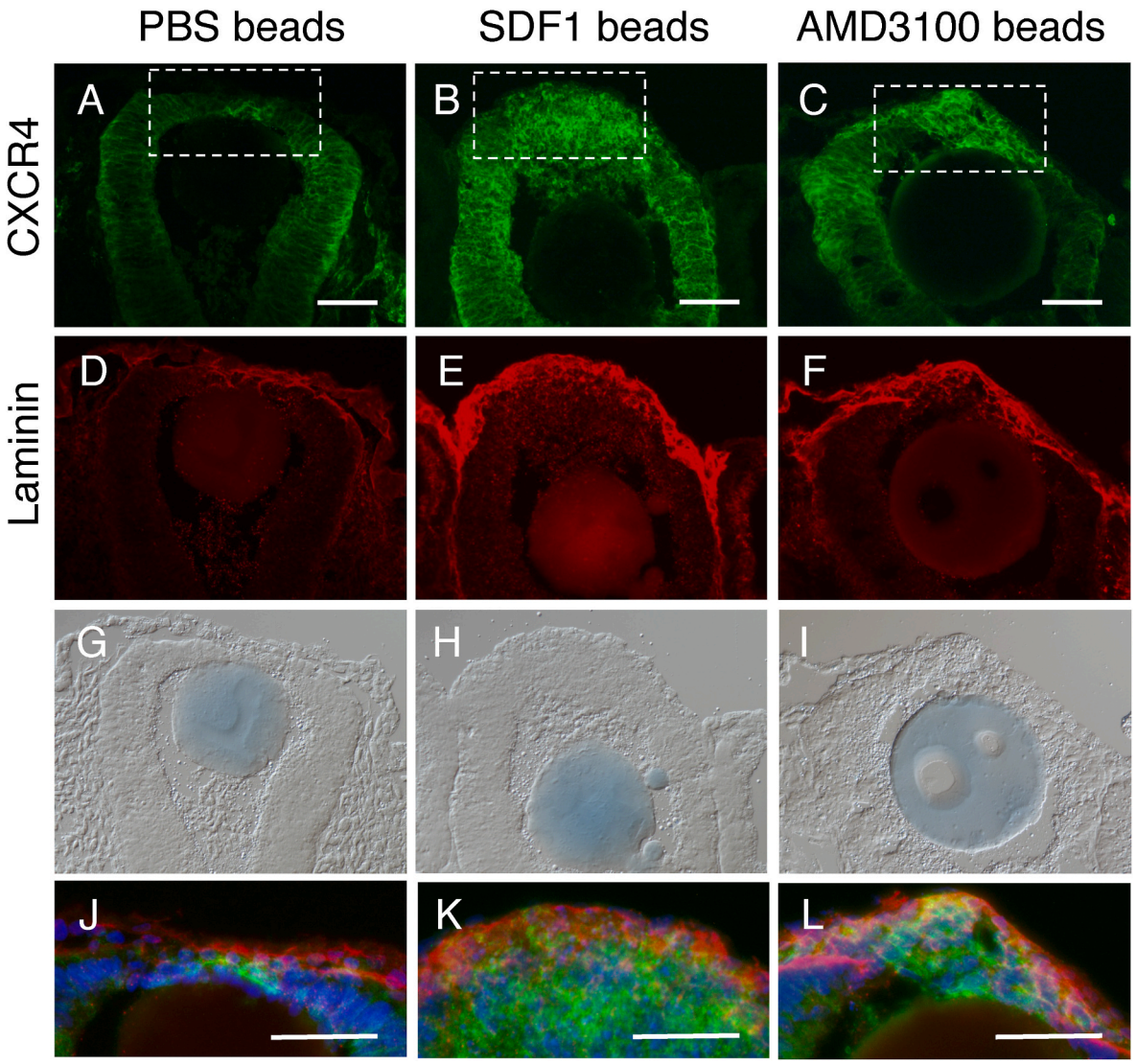

Fig. 3. Disruption of CXCR4/SDF1 signaling affects the formation of the basement membrane in the dorsal neural tube. PBS, SDF1 or AMD3100 bead-implanted embryos were immunostained with anti-CXCR4 (A–C) and anti-laminin (D–F) antibodies at stage HH13 and sectioned at the level of otic vesicle. (G–I) Bright-field images. (J–L) Enlarged views of the dorsal neural tubes in (A–C). All the nuclei were stained with DAPI. N = 3 (J), 3 (K), 2 (L). Scale bars: 50 μm.

3.5. Disruption of CXCR4/SDF1 signaling does not affect neural crest EMT or delamination

To determine whether the EMT process occurs normally in SDF1/CXCR4 signaling-disrupted embryos, we examined the expression of the marker genes Snail2 and Cad6B in premigratory neural crest cells by immunostaining (Fig. 4). It has been previously shown that Snail2 is expressed in premigratory neural crest cells undergoing EMT and the subsequent early migrating neural crest cells [26]. During neural crest EMT, Cad6B is known as a premigratory neural crest marker, and its expression is directly repressed by Snail2 during EMT [4]. A section of the embryos implanted with PBS beads revealed that Snail2-expressing neural crest cells reside in the neural crest region at the dorsal midline of the neural tube, whereas Snail2 expression was barely detectable in the neural crest cells migrating apart from the neural tube (Fig. 4A). In the same transverse section, Cad6B expression was detected in premigratory neural crest cells of the neural tube (Fig. 4D). Snail2 and Cad6B expression was largely reciprocal in neural crest EMT, consistent with a previous report (Fig. 4J) [4]. When the embryo was implanted with SDF1 or AMD3100 beads, Snail2-expressing cells populated around the beads and formed a cell cluster on the dorsal neural tube (Fig. 4B and C). In contrast, Cad6B was not expressed in the accumulated cell cluster in the dorsal midline of the embryos (Fig. 4E and F). We further examined the expression patterns of RhoB in these beads-implanted embryos. RhoB is one of the Rho family GTPase and required for neural crest EMT. In the embryos implanted with PBS beads, the RhoB mRNA expression

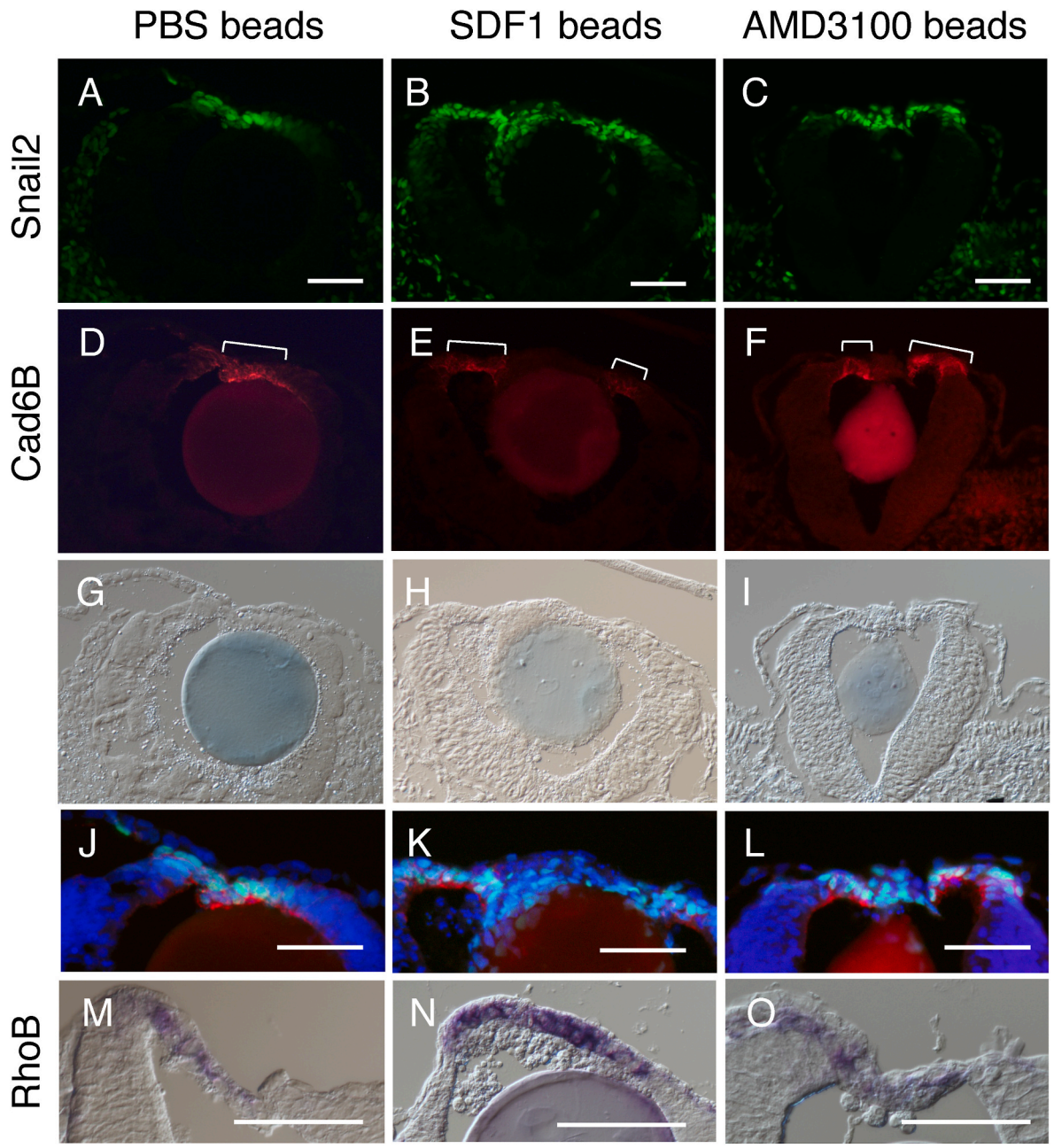

Fig. 4. Disruption of CXCR4/SDF1 signaling does not affect the EMT process. PBS, SDF1 or AMD3100 bead-implanted embryos were immunostained with anti-Snail2 (A–C) and anti-Cad6B (D–F) antibodies at stage HH13 and sectioned at the level of the otic vesicle. (M–O) The detection of RhoB expression in the embryos implanted with PBS, SDF1or AMD3100 beads. (G-I, M – O) Bright-field images. (J–L) Enlarged views of the dorsal neural tubes in (A–C). All the nuclei were stained with DAPI. The brackets show the Cad6B signal regions. N = 2 (J), 3 (K), 3 (L), 3 (M), 3 (N), 4 (O). Scale bars: 50 μm.

was observed in a small population of premigratory neural crest cells and early migrating neural crest cells. This pattern is consistent with that of wild type embryos reported previously [27] (Fig. 4M). In the embryos implanted with SDF1 or AMD3100 beads, the RhoB-expressing domains are expanded in dorsal neural tube (Fig. 4N and O). Thus, we concluded that delaminated neural crest cells accumulate as cell clusters in the dorsal neural tube in embryos with SDF1 or AMD3100 beads. Notably, Cad6B expression was observed at bilateral sites neighboring delaminated neural crest cells in dorsal neural tubes (Fig. 4D–F). These results led us to conclude that EMT sites for delaminating neural crest cells are separated bilaterally due to the accumulation of neural crest cell clusters at the dorsal midline, whereas the EMT process is not affected by the disruption of CXCR4/SDF1 signaling. These results demonstrate that CXCR4-mediated control of cardiac neural crest cells begins from the onset of migration following delamination.

In this study, we show that the initial migration of cardiac neural crest cells is inhibited by the disruption of CXCR4/SDF1 signaling. The previous study by Escot et al., 2013 indicated that CXCR4/SDF1 signaling regulates early cardiac neural crest migration in the dorsolateral pathway to the pharyngeal arches. This finding was obtained by electroporation experiments for the overexpression of SDF1 or loss of function of CXCR4 in the neural tube, which are technically difficult to access specifically in the nascent neural crest cell population [18]. By implantation of SDF1-soaked beads between neural tube and somites or lateral to the somites, it was reported that CXCR4 controls the ventral migration of trunk neural crest cells [28]. Here, we showed that bead implantation into the closing neural tube is an effective way to investigate the initial migration step of cardiac neural crest cells. The

implantation of SDF1- or AMD3100-soaked beads caused cell accumulation in the dorsal neural tube. These accumulated cells, which express *Sox10*, include not only migrating neural crest cells but also massive delaminated neural crest cells. Notably, the delaminated neural crest cells retained Snail2 expression. Thus, we suppose that these cells may be inhibited from undergoing the migration step after delamination. In addition, disruption of CXCR4/SDF1 signaling even with AMD3100 beads led to invasion of cardiac neural crest cells into the dorsal lumen of the neural tube. A similar phenotype was observed when ectopic N-cadherin (Ncad) or Cadherin-7 (Cad7) expression occurred, suggesting that the regulated expression of cadherins in the neuroepithelium may be required for maintaining apicobasal polarity and sending out neural crest cells in the right direction [29]. In our study, we propose that the accumulation of cardiac neural crest cells in the dorsal neural tube may affect the pattern of Ncad or Cad7 expression, resulting in the invasion of cardiac neural crest cells into the lumen due to abnormal apicobasal polarity.

CRediT authorship contribution statement

Saori Tani-Matsuhana: Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Yumi Unozu:** Visualization, Investigation, Data curation. **Kunio Inoue:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2025.151914.

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