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A new role of the membrane-type matrix metalloproteinase 16 (MMP16/MT3-MMP) in neural crest cell migration

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ABSTRACT Neural crest cells (NCCs) are a transient population of neuroectodermal-originated cells that populate the dorsal neural tube (dNT), before migrating and giving rise to multiple cell lineages in the developing embryo. Prior to their migration, NCCs undergo epithelial-to-mesenchymal-transition (EMT) through which they lose cell contacts and detach from the dNT to invade their surrounding environment. Multiple signals and transcription factors have been identified to regulate these events. Yet, less is known regarding effectors that act downstream to execute the actual NCC separation and migration. Matrix metalloproteinases (MMPs) are a family of proteases that degrade the extracellular matrix as well as other pericellular proteins during processes of tissue remodeling, angiogenesis and metastasis. Previously, we and others have demonstrated the role of the gelatinases MMP2 and MMP9 during the onset of NCC migration. Several evidences link the cleavage and activation of these secreted gelatinases to the activity of membrane-type MMPs (MT-MMP), such as MMP14 and MMP16, which are tethered to plasma membrane and affect various cellular behaviors. The aim of this study was to investigate whether MMP16 acts in NCCs. Here we demonstrate the expression of MMP16 mRNA and protein in cranial NCCs in avian embryos. Knockdown of MMP16 inhibited NCC migration. This inhibition was rescued by the addition of recombinant MMP16, which was also sufficient to increase proper NCC migration. Furthermore, excess MMP16 caused enhanced NCC EMT, concomitant with degradation of dNT-related proteins, laminin and N-cadherin. Altogether, these results uncover MMP16 as a new effector participating in EMT and in the migration of NCCs.

KEY WORDS: *chick embryo, dorsal neural tube, EMT, laminin, cadherin.*

Introduction

Neural crest cells (NCCs) compose a transient embryonic cell population formed at the border between the neural plate and the non-neural ectoderm. With neural tube closure, NCCs become located at its dorsal regions, and in a synchronized process from head to tail, they detach from the neural tube and migrate in determined routes throughout the developing body. Upon arrival to their target tissues, NCCs fully differentiate into multiple derivatives such as melanocytes, sensory neurons, glia and Schwann cells, enteric nervous system cells and neuroendocrine cells. Cranial and vagal NCCs also give rise to craniofacial bones and cartilage and to cardiac connective tissues (Barembaum and Bronner-Fraser, 2005; Kulesa and Gammill, 2010; Le-Douarin and Kalcheim, 1999; Takahashi *et al.*, 2013; Trainor, 2005). In order to engage in migration, NCCs undergo epithelial-to-mesenchymal transition (EMT), upon which they become separated and motile. This process requires fundamental changes in the cells and their surrounding environment such as breakage of cell-cell interactions, rearrangement of cell cytoskeleton and remodeling of the extracellular matrix (ECM) (Clay and Halloran, 2011; McKeown *et al.*, 2013; Perris and Perissinotto, 2000; Taneyhill, 2008).

Abbreviations used in this paper: dNT, dorsal neural tube; EMT, epithelial-to-mesenchymal-transition; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; NCC, neural crest cell.

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A prominent trigger of NCC migration is Bone Morphogenic Protein (BMP). Tightly regulated by its inhibitor noggin, BMP induces Wnt signaling, modulates the activity of small GTPases and prompts N-cadherin cleavage to induce separation of NCCs (Burstyn-Cohen *et al.*, 2004; Groysman *et al.*, 2008; Schiffmacher *et al.*, 2014; Sela-Donenfeld and Kalcheim, 1999; Shoval *et al.*, 2007; Shoval and Kalcheim, 2012; Ulmer *et al.*, 2013). A network of transcription factors, such as Forkhead box D3 (Foxd3), Snail and Sox8-10 is also fundamental in this process by inducing or repressing the expression of adhesion molecules, cell cycle genes and cytoskeleton-related proteins (Jhingory *et al.*, 2010; Knecht and Bronner-Fraser, 2002; Minoux and Rijli, 2010; Taylor and LaBonne, 2007). Downstream of these pathways, other types of proteins such as enzymes should be activated to directly execute the degradation of NCC contacts and the adjustments of their microenvironment to allow motility. Knowledge about such effectors is sparse.

The need to modify cell and ECM contacts to allow NCC migration raises the possibility that matrix remodeling enzymes are involved (Bar *et al.*, 2014; Christian *et al.*, 2013; Perris and Perissinotto, 2000). Matrix-metalloproteinases (MMPs) are a family of secreted or membrane bound enzymes, known primarily for their ability to degrade a wide variety of ECM components as well as other pericellular proteins. So far, ~25 different MMPs have been recognized in vertebrates which are divided into four subfamilies according to their main (but not exclusive) substrates: collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs) (Mott and Werb, 2004; Nelson *et al.*, 2000; Somerville *et al.*, 2003). Many studies demonstrated their fundamental role as activators of EMT, motility and invasiveness in tumors. By remodeling the ECM and adhesion proteins, MMPs evacuate space for cell migration and locomotion. They also affect intracellular signaling pathways by exposing or cleaving latent proteins and/or factors trapped in the ECM, by obscuring cell surface proteins and by modulating cell-ECM related interactions which affect the cytoskeleton (Brinckerhoff and Matrisian, 2002; Lee *et al.*, 2005; Orlichenko and Radisky, 2008; Page-McCaw *et al.*, 2007; Vu and Werb, 2000). Of note, some MMPs are also necessary to cleave and activate other MMPs, providing a cascade of well-regulated degradation events that lead to ECM modulation and cell migration (Itoh, 2015).

So far, few MMPs, mostly of the gelatinases subgroup, were reported to be active in migratory NCCs (Bar *et al.*, 2014; Christian *et al.*, 2013); the gelatinase MMP2 was demonstrated to be transiently expressed in avian NCCs, and its inhibition perturbed some phases of NCC migration (Duong and Erickson, 2004). MMP2, together with the MMP inhibitor TIMP2, were also found to be involved at later stages, when NCCs migrate towards the heart and gut (Anderson, 2010; Cai *et al.*, 2000; Cantemir *et al.*, 2004). MMP2 was also demonstrated to be necessary for the migration of NCC-derived melanophores in frogs (Tomlinson *et al.*, 2009). We have recently found a key role of the other gelatinase, MMP9, in executing EMT and migration of cranial and trunk NCCs in chicks, through degradation of laminin and N-cadherin (Monsonego-Ornan *et al.*, 2012).

The MT-MMPs subfamily of MMPs is tightly connected to the development and remodeling of several organs and plays a significant role in promoting cell EMT, migration, neovascularization and metastasis in multiple contexts (Ellenrieder *et al.*, 2000; Hadchouel *et al.*, 2008; Huh *et al.*, 2007; Jalali *et al.*, 2015; Lowy *et al.*, 2006; Tatti *et al.*, 2015; Yang *et al.*, 1996). Additionally to their ability to cleave ECM proteins, MT-MMPs, such as MMP14 and MMP16, were also found to cleave the pro-domain of the gelatinases MMP2 and MMP9 in different cell types, which is an essential step for their activation (Harrison *et al.*, 2004; Hasebe *et al.*, 2007; Itoh, 2015; Sato *et al.*, 1996; Shofuda *et al.*, 2001; Walsh *et al.*, 2007; Zhao *et al.*, 2004). Notably, MMP14-null mice and MMP14/16-double knockout mice present craniofacial defects (amongst other malformations), which may imply on the roles of MT-MMPs in cranial NCC morphogenesis (Holmbeck *et al.*, 1999; Shi *et al.*, 2008). In *Xenopus laevis* embryos, knockdown of MMP14 prevented melanoblast migration, whereas MMP16 overexpression led to neural and head abnormalities, further suggesting their possible involvement in NCC development (Hammoud *et al.*, 2006; Tomlinson *et al.*, 2009). In this study we directly examined the expression pattern and role of MMP16 (MT3-MMP) in triggering the avian NCC EMT and migration.

Results

MMP16 is expressed in migrating cranial neural crest cells

The MT3-MMP/MMP16 was shown to cleave and activate the gelatinases MMP2 or MMP9 in several cell types (Ellenrieder *et al.*, 2000; Hahn-Dantona *et al.*, 2000; Itoh, 2015; Malemud, 2006). These gelatinases were previously found to be involved in executing NCC migration (Cai *et al.*, 2000; Cantemir *et al.*, 2004; Duong and Erickson, 2004; Monsonego-Ornan *et al.*, 2012). Since MMP16 has not yet been reported in NCCs, we set to determine whether it is expressed and active in migrating NCCs in the chick embryo. We first tested its expression in various embryonic stages and axial levels at which NCC migration occurs by using RT-PCR (Fig. 1A). RNA was extracted from pools of whole embryos of 10, 16, and 25 somites, stages when NCCs migrate from cranial, vagal and trunk neural tube, respectively (Fig. 1Aa). RNA was also extracted from *ex-vivo* explants obtained from hindbrain levels of 5-8 somite-stage (ss) embryos, before or after culturing the neural tubes on fibronectin-coated dishes, as well as from NCCs that detached from the neural tube explant and migrated away in the dish (Fig. 1Ab). *MMP16* transcripts were found in embryos from all examined stages as well as in the neural tube and migrating NCCs.

To further characterize *MMP16* expression, *in-situ* hybridization (ISH) was performed on whole embryos and on sections, using MMP16-specific RNA antisense probe (Fig. 1B). *MMP16* staining was compared to *FoxD3,* a typical marker which labels migratory NCCs (Kos *et al.*, 2001). *MMP16* was found to be expressed in cranial NCC streams during their migration, as shown in the midbrain and hindbrain regions of 15ss embryo (Fig. 1Ba). Transverse tissue sections validated *MMP16* expression in detaching NCCs at the dNT as well as during their ventral migration (Fig. 1B c,d). Noticeably, *FoxD3* was also found in migratory NCCs in midbrain and hindbrain axial levels at the same stages (Fig. 1B b,e). The resemblance of *FoxD3* staining to that of *MMP16* further validated the identity of the stained cells as NCCs.

To confirm the presence of MMP16 protein in NCCs, we next examined embryos using an antibody against MMP16. Sections were taken from hindbrain and cervical levels of 16ss embryos (Fig. 1C). MMP16 was evident in migratory NCCs, as shown by co-staining with the classical NCC marker HNK-1 (Fig. 1C). MMP16 distribution was also examined in NCCs grown *ex-vivo*: explants of neural primordia that contain pre-migratory NCCs were dissected

from hindbrain levels of embryos of 5-8ss and cultured in fibronectin-coated dishes. After 16 hours of incubation migratory NCCs were evident around the neural tube explants (Fig. 1Da). Immunostaining revealed many migrating NCCs that express MMP16 protein in the cytoplasm/membrane, as compared to the labeling with phalloidin, an actin-binding dye, which labels cell stress-fibers (Fig. 1D b-d). Altogether, these analyses demonstrate the expression of MMP16 mRNA and protein in migrating cranial NCCs of the chick embryo, suggesting a putative role of MMP16 in this process.

Knockdown of MMP16 inhibits the migration of NCCs

To determine whether MMP16 is necessary for NCC migration, we inhibited MMP16 production *in-vivo*. MMP16 protein was knocked-down using fluorescein-conjugated Morpholino antisense oligonucleotides (MO) (Weisinger *et al.*, 2008), that were directed against the 5'-UTR of MMP16 sequence to inhibit its translation. MMP16-MO and control-MO were unilaterally electroporated into the neural tube of 5-8ss embryos. Immunostaining with HNK-1 was performed 15 hours later to detect the migration of NCCs in MO-treated embryos in whole mount preparations and transverse sections. Control embryos maintained a typical migration pattern of HNK-1$^+$ cranial NCCs, that was evident both at the electroporated and the contralateral sides of the neural tube (Fig. 2 A-A",D, n=24); in contrast, embryos electroporated with MMP16-MO demonstrated a noticeable reduction in migrating cranial NCCs with fewer streams of cells (Fig. 2 B-B",E, n=30). Measurement of HNK-1 staining revealed ~80% reduction in migratory NCC/HNK1$^+$ areas upon MMP16-MO treatment compared to embryos treated with control-MO (Fig. 2G, n= 4 embryos). Noticeably, although MO-antisense oligonucleotides were electroporated unilaterally, the reduction in migration was evident at both sides of the neural tube. This may be a result of MO-electroporation occurring at much lower levels also in the contralateral neural tube, which is not detected by the staining, yet sufficient for downregulating the target gene (Weisinger *et al.*, 2008). Alternatively, as MMP16 may activate or degrade other soluble MMPs, such as MMP9 and MMP2 (Itoh, 2015; Walsh *et al.*, 2007; Zhao *et al.*, 2004), which are known to affect NCC migration (Duong and Erickson, 2004; Monsonego-Ornan *et al.*, 2012), it is possible that they act in the ECM in a limiting concentration and that MMP16 loss-of function causes them to be less available also in areas outside of the knockdown cells.

To further confirm these data, a rescue experiment was performed. Embryos of 5-8ss were electroporated with MMP16-MO as described above, and left to develop for six hours. Next, the embryos were injected with 4ng/μl soluble MMP16 into the neural tube. Following further incubation of 10 hours, embryos were analyzed for cranial NCC migration. Administration of soluble MMP16 to the morphants reverted the MMP16-MO effect and resulted in a normal migration pattern of NCCs (Fig. 2 C-C",F, n=7). The rescue of MMP16-MO treated embryos upon exogenous MMP16 further supports the role of MMP16 in NCC migration, in agreement with the results obtained in MMP16 gain-of-function experiments (Figs.3,4). This experiment also rules out the possibility of a toxic effect of the MO treatment on NCC development. This was also confirmed by the normal morphology of the different embryos seen in bright-field views (Fig. 2A-C), as well as by the similar

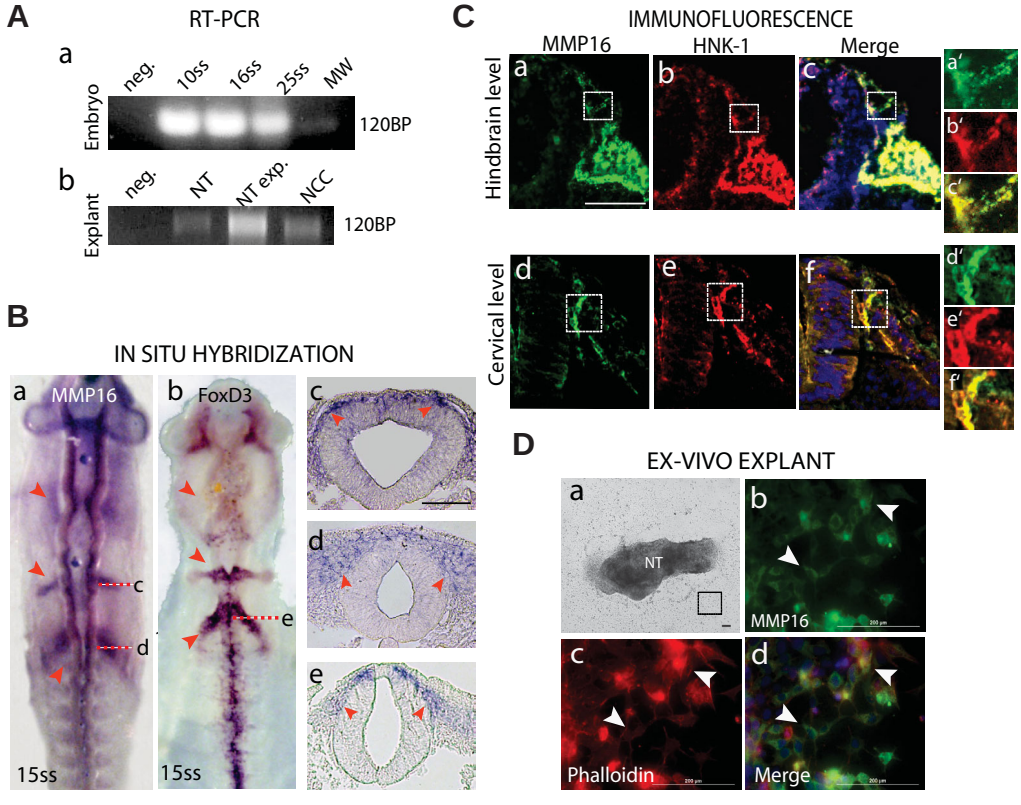

Fig. 1. MMP16 is expressed in neural crest cells (A) *RT-PCR analysis on mRNAs purified from whole embryos of 10, 16, 25 somites (a) or from neural-tube explants obtained from hindbrain levels of 5-8ss embryos (b), either before (NT) or after (NT exp.) culturing in* ex-vivo *conditions, and from NCCs that migrated away from the explants (NCC) (b). Primer pairs were directed against a 120bp long sequence of chick MMP16. Negative control in (a,b) represents reaction buffer without cDNA.* **(B)** In-situ *hybridization on whole-mounts (a,b), or paraffin sections (c-e) of 15ss embryos labeled with RNA probes against MMP16 (a,c,d) or FoxD3 (b,e). Paraffin sections (c-e) were taken from the dotted lines in (a,b). Red arrowheads indicate NCCs which express MMP16 (a,c,d) or FoxD3 (b,e).* **(C)** *(a-f) Immunolabeling of transverse sections obtained from hindbrain (a-c) or cervical (d-f) axial levels from 15ss embryos using MMP16 (a,d) and HNK-1 (b,e) antibodies. Images show half of the neural tube. Merged images of both stains are shown in (c,f), together with nuclear staining (DAPI, blue). Panels (a'-f') are enlargement of the boxed areas in panels (a-f).* **(D)** *(a-d) Staining of neural tube explants with MMP16 antibody (b) or phalloidin (c). Bright field view is shown in (a). Panels (b-d) are an enlargement of the boxed area in panel (a). Merged image of both staining is shown in (d). White arrowheads indicate MMP16-expressing NCCs. In all panels, stages and staining are indicated. NT, neural tube; ss, somite-stage; neg, negative control; NT exp, neural tube explant; NCC, migratory NCCs; MW, molecular weight marker. Bars in B(c-e) and C(a-f) 100 μm, in D(a) 10 μm, in D(b-d) 200 μm.*

distribution of dividing or apoptotic cells in embryos treated with either control or MMP16-MOs, as analyzed by phospho-histone 3 immunostaining and TUNEL assay, respectively (Fig. 2 H-K, n=6 for each treatment).

Addition of exogenous MMP16 accelerates NCC migration

In a parallel approach, we determined the effect of excess MMP16 on NCC migration. Embryos of 5-8ss were injected with control conditional media or media supplemented with 4ng/μl MMP16. Media was injected twice into the rostral neural tube with three hour incubation intervals. Embryos were harvested 8 hours after the first addition, at the stage of 10-12 somites, and stained with HNK-1 antibody. Typical migration of NCC streams was evident in whole-mounted embryos receiving control media, as expected by the time of fixation (Fig. 3A, arrowhead, n=16). A remarkable increase in HNK-1$^+$ NCCs was observed in embryos treated with MMP16-supplemented media (Fig. 3B, arrowhead, n=16). Moreover, embryos treated with MMP16 showed precocious migration of NCCs from the caudal hindbrain, in contrast to controls where NCCs were not yet engaged in migration at this axial level (Fig. 3 A,B, arrows). Transverse sections taken from control and MMP16-treated embryos confirmed these results, showing augmentation of migrating NCCs in MMP16-treated embryos, compared to the controls (Fig. 3 C,D, arrowhead). ISH staining was also applied on similarly treated embryos using a probe against *FoxD3*. Whole mounted and sectioned embryos revealed enhancement in *FoxD3* distribution in MMP16-treated embryos in comparison to controls (Fig. 3 E-H, arrows; control n=7, MMP16 n=8). Quantification of the FoxD3$^+$ areas was performed in transverse sections, revealing ~56% increase in *FoxD3*$^+$ NCC streams in the MMP16-treated embryos compared to controls (Fig. 3I, n= 5 embryos for each group). Altogether, these results demonstrate that excess MMP16 is sufficient to enhance NCC migration from the neural tube *in-vivo*.

We next examined whether exogenous MMP16 can increase NCC migration in neural tube explants that are devoid of surrounding tissues, as opposed to the *in vivo* experiments. Control medium or medium supplemented with 0.4ng/μl MMP16 were added to neural tube explants that were isolated from hindbrain levels

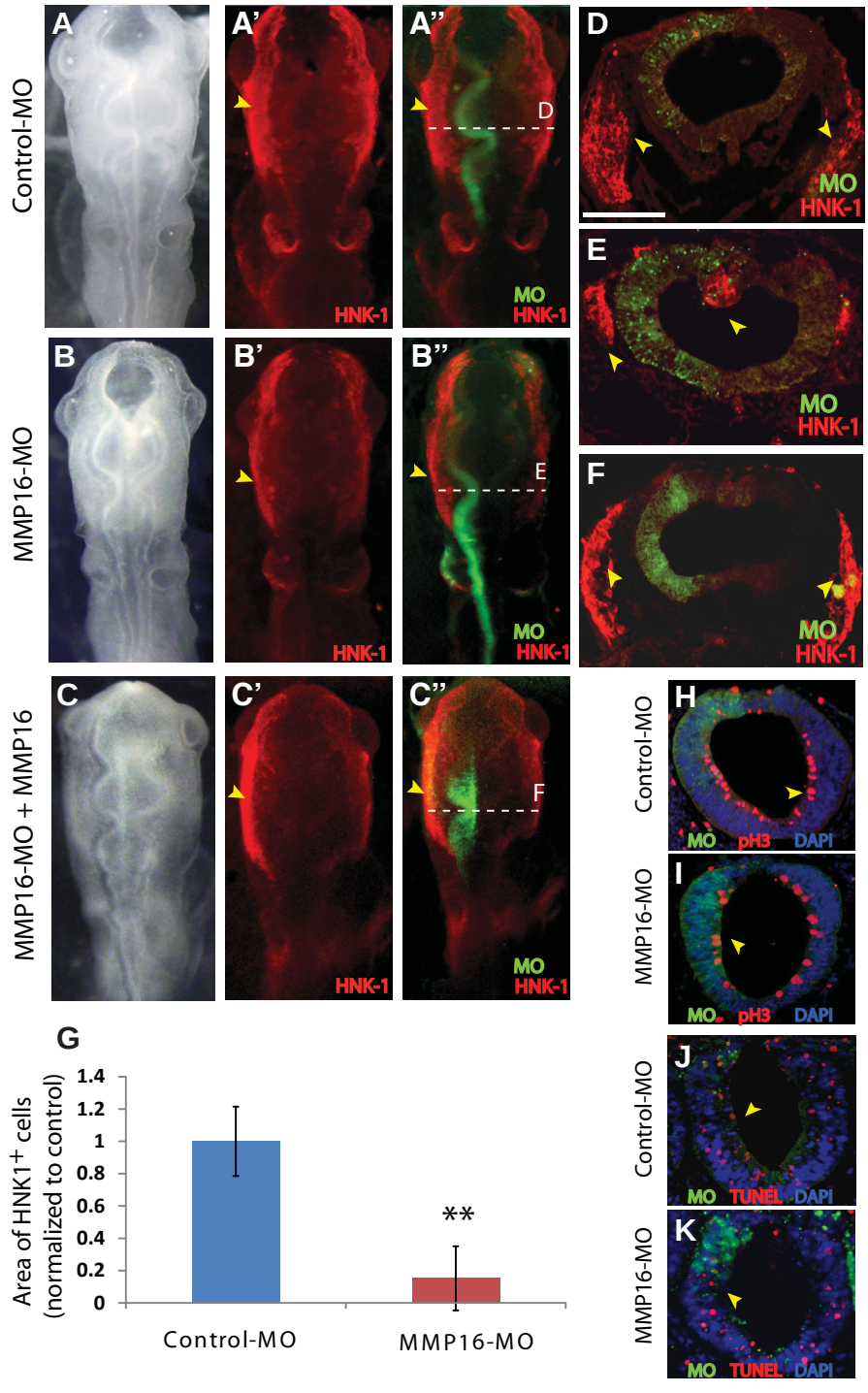

Fig. 2. Knockdown of MMP16 inhibits neural crest cell migration. (A-C") *Whole-mount images of bright-field (A-C) or HNK-1-stained embryos (A'-C") that were electroporated at the stage of 5-8 somites with fluorescein-conjugated control-MO (A) or MMP16-MO (B,C). Embryo in (C) was also injected with MMP16-enriched media six hours after the electroporation. Panels (A"-F) are a merge of HNK-1 (red) and MO-fluorescein (green) staining. Yellow arrowheads in (A'-C") mark HNK-1*$^+$ *NCCs.* **(D-F)** *Transverse sections taken from the dotted lines in (A"-C"), respectively.* **(G)** *Quantification of migratory HNK-1*$^+$ *cells obtained from transverse sections of embryos treated with MMP16-MO relative to control-MO treated embryos. Cells were measured from the electroporated side. Bars represent mean and standard deviation (** P<0.005).* **(H-K)** *Transverse sections of control-MO or MMP-16 MO electroporated embryos stained for pH3 (H,I) or TUNEL (J,K). Yellow arrowheads represent pH3*$^+$ *or TUNEL*$^+$ *cells, respectively. In all images, treatments and staining are indicated. Bars in (D,F, H-K) 100 μm.*

of 5-8ss embryos. Explants were cultured for 16 hours and the migration of NCCs around the explants was examined following HNK-1 staining (Fig. 4 A-D). As expected, in control explants NCCs detached from the neural tube and migrated away (Fig. 4 A,C, dotted black/white circle respectively, n=12; see also Fig. 4 F,H). However, addition of MMP16 enhanced the area occupied by the detaching NCCs (Fig. 4 B,D, dotted black/white circle respectively, n=12; see also Fig. 4 G,I). Remarkably, excess MMP16 did not affect the integrity of the neural tube (Fig. 4A-D), indicating that MMP16 acts to enhance NCC delamination from the neural tube rather than to induce general cell dissociation in culture. Quantification of these results revealed ~40% increase in the area occupied by migrating NCCs in the presence of excess MMP16 as compared to control (Fig. 4E, n=5 explants).

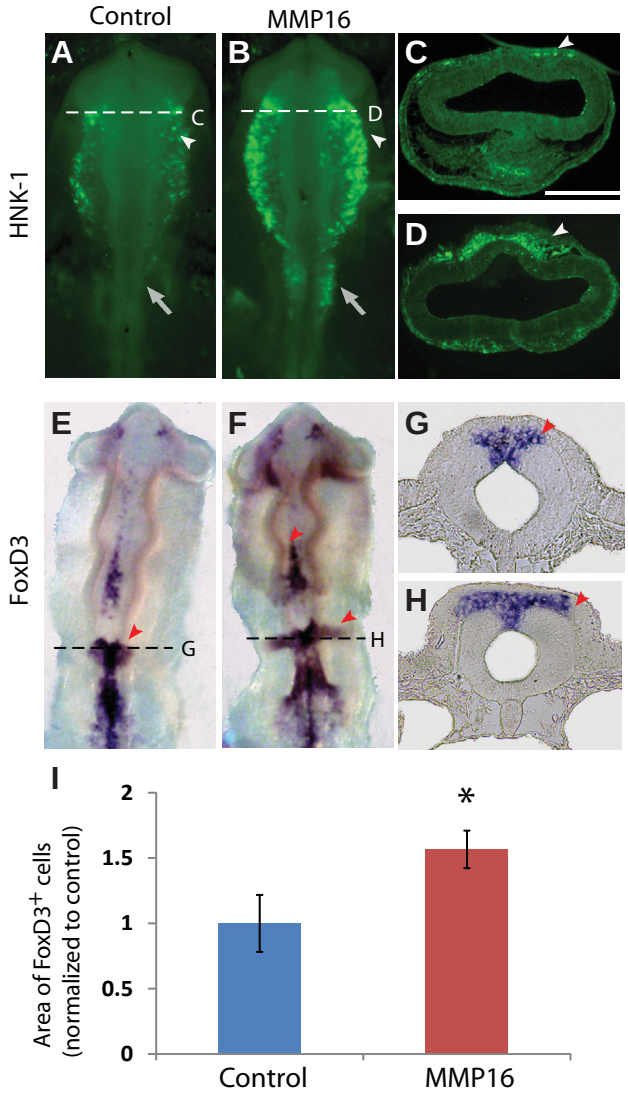

Fig. 3. Excess MMP16 enhances neural crest cell migration. (A,B,E,F) *Whole mount views of embryos injected with control (A,E) or MMP16-containing media (B,F) and processed 6 hours later for HNK-1 immunostaining (A,B) or for* in-situ *hybridization with FoxD3 probe (E,F).* **(C,D,G,H)** *Transverse sections taken from dotted lines in (A,B,E,F), respectively.* **(I)** *Quantification of migratory* FoxD3$^+$ *cells from transverse sections upon injection with MMP16 containing media relative to control media. Bars represent mean and standard deviation (* $P<0.05$*). In all images, treatments are indicated. White arrowheads and arrows mark HNK-1-expressing NCCs in anterior or vagal regions, respectively. Red arrowheads mark* Foxd3*-expressing NCCs. Bars in (C,D,G,H) 100* μ*m.*

A question raised by these results is how MMP16 affects NCC migration. As NCCs undergo EMT in order to engage in migration, the impact of MMP16 was next examined on the epithelial/mesenchymal characteristics of NCCs. Explants were analyzed for their morphology and organization of actin stress fibers by staining with phalloidin (Groysman *et al.*, 2008; Wright *et al.*, 1988). Adjacent to the neural tube, control explants maintained the typical pattern of 'epithelioid' sheet, which reflects early-migrating NCCs of an intermediate morphology between initial epithelial and subsequent mesenchymal phenotype (Newgreen and Minichiello, 1995). These cells maintain cell-cell contacts, organized stress fibers and a cubical-like shape (Fig. 4 F,F', n=7). In contrast, addition of MMP16 led to separation of NCCs shortly after their migration, with disruption of their organized stress fibers and gain of triangular morphology, which is typical of mesenchymal cells (Fig. 4 G,G', n=6). Notably, at distance from the NT, control NCCs also showed a more mesenchymal-like phenotype, as expected in normal conditions (Fig. 4F''). Yet, MMP16-treated NCCs at a similar distance from the NT looked more mesenchymal and separated from each other (Fig. 4G'').

The epithelial/mesenchymal properties of the cells were also examined by staining for N-cadherin, an adhesion molecule expressed in the apical surface of the neural tube which is being downregulated from its dorsal domains during NCC migration (Groysman *et al.*, 2008; Nakagawa and Takeichi, 1998; Shoval *et al.*, 2007; Taneyhill, 2008). Staining of control explants revealed a typical membrane N-cadherin localization in the epithelioid NCCs around the neural tube (Fig. 4 H-H'', n=8), and its expected downregulation in separated cells that moved further away (data not shown). Yet, N-cadherin was readily absent in the migratory NCCs of the MMP16-treated explants, regardless of their distance from the neural tube (Fig. 4 I-I'', n=8). These *in-vivo* and *ex-vivo* data strongly suggest that MMP16 is sufficient to enhance NCC separation and migration, as evident by the increased number of migrating NCCs and switch from epithelial to mesenchymal morphology. Notably, although MT-MMPs contain transmembrane domains, previous studies have shown that some MT-MMPs can be shed from cell membranes into a soluble active form to gain access to a larger variety of substrates, suggesting additional layer of regulation on MT-MMP's activity (Osenkowski *et al.*, 2004). Concomitantly, purified active MMP16, which lacked its transmembrane domain, cleaved a large variety of ECM components *in vitro* (Shimada *et al.*, 1999) and was also shown in *Xenopus laevis* to be more potent compared to the full-length form (Walsh *et al.*, 2007). As we use recombinant MMP16, it is also possible that some of the observed effects on NCCs were amplified by using soluble MMP16. Yet, the lack of any broad cell-degradation effect in the embryos and explants upon the addition of soluble MMP16 (Figs. 3-5), together with the prevention of NCC migration in MMP16 loss-of-function assay (Fig. 2), further supports an endogenous role of MMP16 in this process.

Degradation by N-cadherin and laminin is mediated by MMP16

N-cadherin was suggested to be regulated by, or to be a substrate of, MT-MMPs in mast cell-neuron interactions and in neural stem

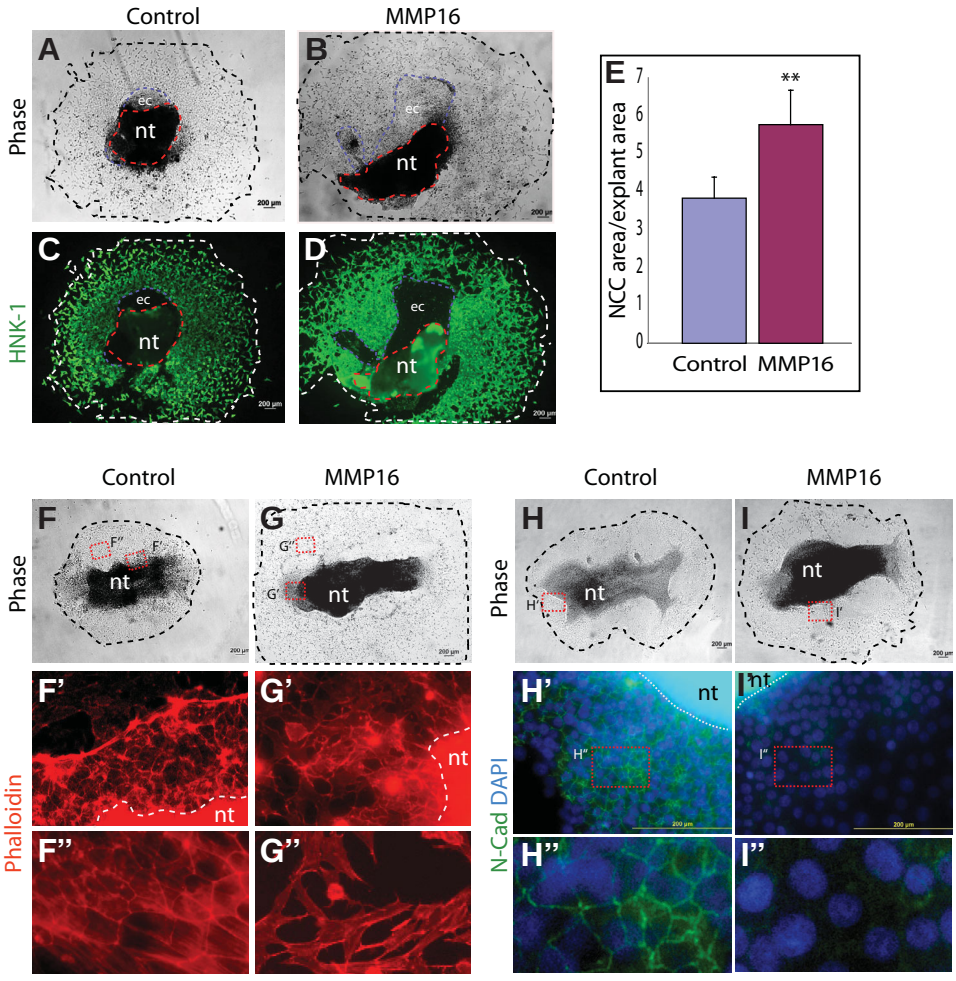

Fig. 4. MMP16 enhances epithelial-to-mesenchymal-transition (EMT) and migration in neural crest cell explants. (A-D) *Bright field views (A,B) or HNK-1-immunolabelled images (C,D) of isolated neural tubes from hindbrain levels that were grown for 16 hours in control (A,C), or MMP16-containing media (B,D). Dashed black/white circles represent borders of the area occupied by migrating NCCs. Dashed red and blue areas represent the NT-explant area (red) and the area occupied by remaining ectoderm (non-neural crest tissue, blue).* **(E)** *Quantification of migratory NCC areas upon addition of MMP16 media versus control explants. Bars represent mean and standard deviation (** P<0.005).* **(F-I")** *Views of bright field (F-I), Phalloidin-stained (F',G') or N-Cadherin immuno-labelled (H',I') explants treated with control (F,H) or MMP16-enriched media (G,I). Images (F'-G") are enlargements of the red-boxed areas in (F,G); Panels (F',G') are enlargements of early migrating NCC close to the NT; Panels (F",G") are enlargements of farer areas of advanced migrating NCCs. Images (H',I') are enlargements of the red-boxed areas in (H,I) and images (H",I") are enlargements of the red-boxed areas in (H',I'). In panels (F-I) dashed black/white circles represent borders of the area occupied by migrating NCCs. Dashed lines in panels (F'-I') represent the neural tube. In all images, treatments and staining are indicated. nt, neural tube; ec, ectoderm; N-Cad, N-cadherin. Bars 200 μm.*

cells (Folgueras *et al.*, 2009; Itoh, 2015; Porlan *et al.*, 2014). While N-cadherin expression was decreased in early-migrating NCCs upon exposure to MMP16 (Fig. 4), the over-night incubation period with MMP16 does not allow to determine whether N-cadherin is a direct MMP16 substrate or whether its downregulation is a consequence of a boosted onset of NCC migration. We have previously found that transient incubation of NCCs with MMP9 is sufficient to degrade N-cadherin protein, suggesting a direct effect of MMP9 on N-cadherin degradation (Monsonego-Ornan *et al.*, 2012). To test whether N-cadherin is also a potential direct target of MMP16, neural tube explants were first incubated in control media for 16 hours to allow normal detachment and migration of NCCs. Subsequently, explants were exposed to MMP16 for 3 hours and compared to control explants (Fig. 5 A-F). In all groups, the migration area of NCCs around the neural tube was similar, as MMPs were added only for a short period. Also, in all treatments NCCs showed typical epithelioid sheet adjacent to the neural tube, further indicating for the insufficiency of the short exposure to MMPs to cause evident EMT (Fig. 5 A,B,D,E) (see also Monsonego-Ornan *et al.*, 2012). Yet, staining for N-cadherin displayed major differences between treatments: a typical membrane staining of N-cadherin was found in the epithelioid layer of NCCs in controls (Fig. 5 C-C', n=18). However, MMP16-treated explants showed a loss of N-cadherin in the epithelioid NCCs, except for a few discontinuous staining that remained (Fig. 5 F-F', n=15). Similar loss of N-cadherin was also found in MMP9 treated explants, as reported previously (data not shown, see Monsonego-Ornan *et al.*, 2012). The decreased protein levels of N-cadherin in emigrating NCCs, before their separation and conversion into mesenchymal cells, supports the possibility that MMP16 either acts on N-cadherin directly or rapidly activates a downstream protease that in turn cleaves N-cadherin.

To continue determining the effect of MMP16 on N-cadherin *in-vivo*, 5-8ss embryos were injected with MMP16 to gain enhancement of NCC migration, as described above (Fig. 3). Embryos were then sectioned at the hindbrain level and analyzed for N-cadherin staining. Expression of N-cadherin decreased in the dorsal-most part of the apical neural tube of control embryos, at typical axial levels where NCCs leave the neuroectoderm (Fig. 5G,G', arrows, n=6). Addition of MMP16 resulted in a broader area that lacks N-cadherin in the neural tube (Fig. 5 H,H', n=6). Quantification of these results revealed 2.2 fold increase in the area lacking N-cadherin in the MMP16-treated embryos compared to controls (Fig. 5K, n=5 for each group). Concurrent with the *ex-vivo* results (Fig. 4H,I; Fig. 5 A-F), this experiment demonstrates that exogenous MMP16 leads to rapid N-cadherin degradation, which in turn allows the detachment of the cells. Yet, whether MMP16 cleaves N-cadherin directly or indirectly (i.e, by activating another protease such as MMP9) remains to be elucidated.

The secreted ECM protein laminin is a major component of the basement membrane that circulates the neural tube. Yet, when NCC emigration takes place, laminin becomes scattered from its dorsal domain, forming a passage for delaminating NCCs (Desban *et al.*, 2006; Duband and Thiery, 1987). As MT-MMPs were shown to cleave laminin in different contexts (Itoh, 2015; Ohuchi *et al.*, 1997; Shimada *et al.*, 1999), we examined whether MMP16 has an effect on laminin. Embryos were injected with control or MMP16-enriched media as described above, and immunostained for laminin. Control embryos demonstrated typical staining around the basal neural tube, with a gap at its dorsal-most part, where migrating NCCs can be seen (Fig. 5I, I', arrows, n=5). In contrast, addition of MMP16 resulted in a significant widening of laminin-excluded areas in the dNT together with many more NCCs accumulating above this domain (Fig. 5 J,J', arrows, n=5). Quantification of these data showed ~2 fold increase in laminin-negative areas around the basal dNT in the MMP16-treated embryos compared to controls (Fig. 5K, n=5 for each treatment). These data suggest that MMP16 affects the degradation of laminin in the regions where NCC migration occurs, indicating for it as a direct or indirect target of MMP16 proteolytic activity.

Discussion

This study revealed that MMP16 is expressed in cranial NCCs and required for their migration in the chick embryo. MMP16 was also found to enhance NCC separation and mesenchymalization, which was coupled with enhanced degradation of N-cadherin and laminin.

Very little knowledge exists regarding MT-MMPs in NCCs. Ex-

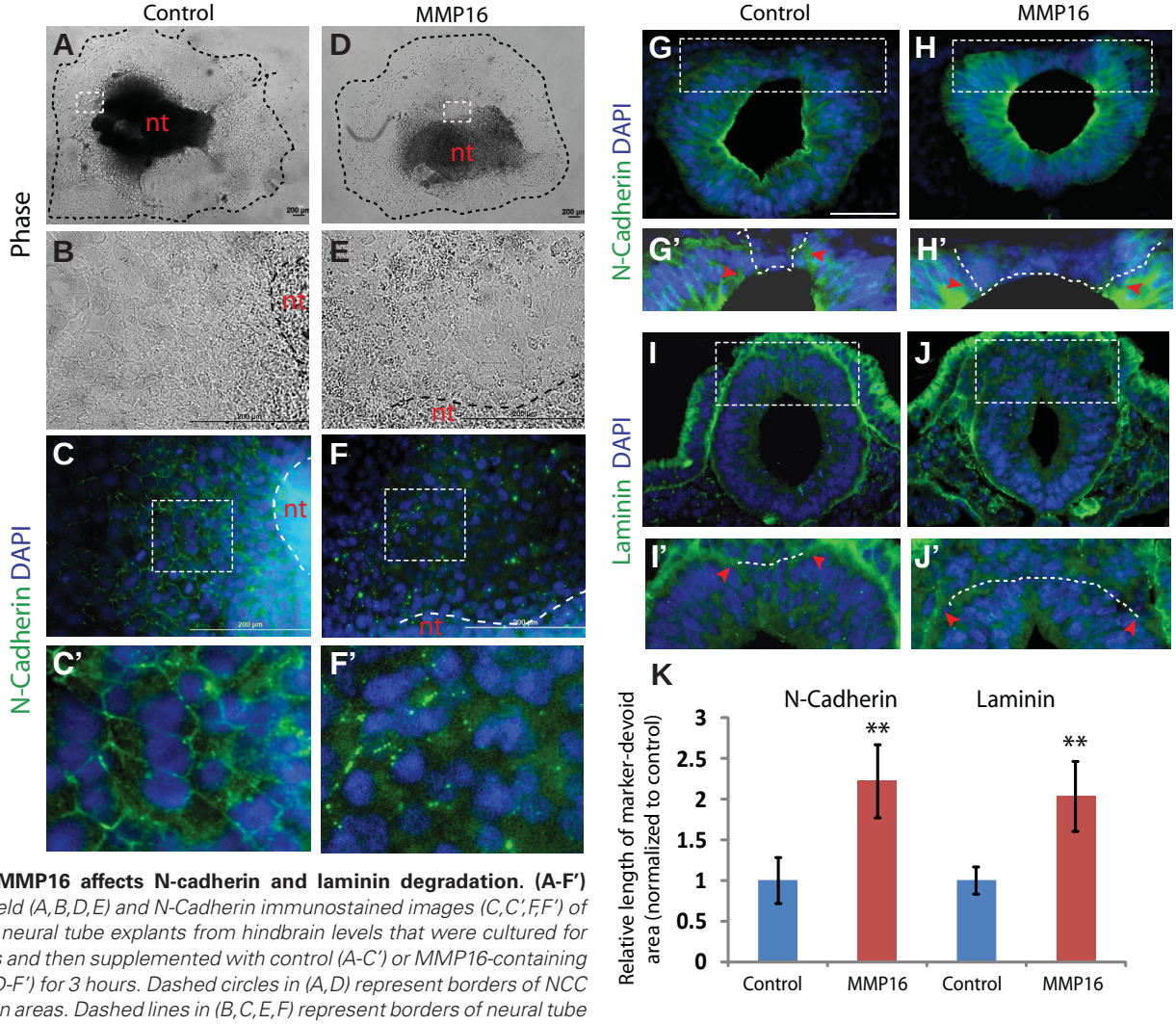

Fig. 5. MMP16 affects N-cadherin and laminin degradation. (A-F') *Bright field (A,B,D,E) and N-Cadherin immunostained images (C,C',F,F') of isolated neural tube explants from hindbrain levels that were cultured for 16 hours and then supplemented with control (A-C') or MMP16-containing media (D-F') for 3 hours. Dashed circles in (A,D) represent borders of NCC migration areas. Dashed lines in (B,C,E,F) represent borders of neural tube explants. Images in (B,E) are enlargements of boxed areas in (A,D), respectively. Images in (C',F') are enlargements of boxed areas in (C,F), respectively.* **(G-J)** *Transverse sections taken from cranial levels of embryos injected with control (G,I) or MMP16-enriched media (H,J). Images in (G'-J') are enlargements of the boxed areas in (G-J), respectively. Sections were stained for N-Cadherin (G-H') or laminin (I,J'). Dashed lines in (G'-J') mark the gap in N-cadherin or laminin expressing areas, respectively. Red arrowheads in (G'-J') indicate N-cadherin or laminin borders of expression in the apical or basal dorsal neural tube, respectively.* **(K)** *A graph representing the effect of MMP16 media versus control media on N-cadherin and laminin distribution in the dorsal neural tube. Bars represent mean and standard deviation (** P<0.005). In all images, blue represents DAPI staining and treatments are indicated. nt, neural tube. Bars in (A-F) 200 μm, in (G-J) 100 μm.*

pression pattern studies demonstrated the possible expression of MMP15 (MT2-MMP) in chick and fish NCCs, amongst other embryonic tissues (Patterson *et al.*, 2013; Quick *et al.*, 2012). Knockdown of MMP14 in frogs or MMP17 (MT4-MMP) in zebrafish showed defects in the migration routes and/or differentiation of NCCs (Leigh *et al.*, 2013; Tomlinson *et al.*, 2009). Moreover, overexpression of MMP16 in frog embryos led to head abnormalities (Hammoud *et al.*, 2006), which may result partially from abnormal development of NCCs. In mice, deletion of MMP16 displayed abnormal skull with subtle growth retardation, whereas MMP14/MMP16 double mutants demonstrated severe craniofacial defects along with other major impairment in collagenolytic abilities (Shi *et al.*, 2008). Whether these phenotypes also correspond to early interference in NCC development in mammals is not known. Yet, all these studies imply for a direct or indirect effect of membrane-bound MMPs on actively-migrating or advanced differentiating NCCs. Our study is the first to demonstrate a role for MT-MMPs in promoting the onset of NCC migration by affecting their EMT.

The effect of MMP16 on NCC EMT and migration is compatible with its roles in tumors. MMP16 was shown to be associated with EMT, metastasis and poor prognosis in different cancers, such as glioblastoma, fibrosarcoma, melanoma, bladder, breast, gastric and colon cancer cells. In many of these studies, MMP16 was found to cleave various types of ECM proteins (i.e, collagen isoforms, gelatin, laminin, fibronectin, vitronectin, fibrin), as well as to activate other proteases, such as proMMP2/9, that in turn promote invasiveness (Cao *et al.*, 2016; Daja *et al.*, 2003; Ellenrieder *et al.*, 2000; Hotary *et al.*, 2006; Kang *et al.*, 2000; Köhrmann *et al.*, 2009; Lowy *et al.*, 2006; Marco *et al.*, 2013; Sato and Takino, 2010; Shi *et al.*, 2008; Shimada *et al.*, 1999; Tatti *et al.*, 2015; Zhao *et al.*, 2004). Concomitant with these findings, downregulation of MMP16 by microRNAs significantly reduced the migration and invasion of glioblastoma and breast cancer cell lines (Liu *et al.*, 2015; Xia *et al.*, 2009).

The various modes of action of MMP16 in cancer cells suggest several scenarios (which are not mutually exclusive) regarding its function in promoting NCC migration; it may act directly on the ECM and basement membrane around NCCs and/or on NCC-adhesion proteins, leading to their EMT and separation. It may also cleave and activate both proMMP2 and MMP9 directly, or act as an activator of proMMP2, which in turn activates proMMP9, enabling these gelatinases to execute NCC detachment and migration (Fridman *et al.*, 1995; Walsh *et al.*, 2007; Zhao *et al.*, 2004). Interestingly, in aggressive melanoma, MMP16 was shown to degrade MMP14 and thereby inhibiting MMP14-dependent collagen invasion (Tatti *et al.*, 2011). As MMP14 was found to affect NCC-derived melanocytes migration in the frog embryo (Tomlinson *et al.*, 2009), future studies are required to determine whether MMP16 act as a modifier of MMP14 activity in NCCs.

Which factor(s) may regulate the expression of MMP16 in NCCs? Studies in gastric cancer cells found that up-regulation of β catenin/Wnt signaling triggers MMP16 expression, which is coupled with increase in invasiveness of neoplastic cells (Lowy *et al.*, 2006). In the growth plate, Wnt/β catenin signaling was similarly found to induce MMP16 expression (along with other MMPs), as a mean to promote chondrocyte maturation and function (Tamamura *et al.*, 2005). Wnt signaling is indispensable for NCC EMT and migration in multiple species (Burstyn-Cohen *et al.*, 2004; Mayor and Theveneau, 2014; Ulmer *et al.*, 2013; Willems *et al.*, 2015). Hence, MMP16 gene expression may also be a downstream target of Wnt signaling in migratory NCCs. Additional putative regulators of MMP16 are the Snail transcriptional regulators. Depletion of Snail1 was found to abrogate MMP16-dependent collagen invasion in fibroblasts (Rowe *et al.*, 2009), whereas ectopic expression of Snail1 in breast cancer cell upregulated the expression of MMP14 and MMP15, leading to enhanced basement membrane invasion (Ota *et al.*, 2009). Since Snail has been demonstrated to induce EMT in NCCs by down regulating E-cadherin (Aybar *et al.*, 2003; del Barrio and Nieto, 2002; Carver *et al.*, 2001), future studies are required to examine whether it also upregulates MMP16 in NCCs. Moreover, ADAM12, a member of the disintegrin and metalloproteinase (ADAM) family of enzymes, was recently found to increase the expression of MMP14 on the cell surface of HEK293 cells, as well as to promote MMP14 dependent ECM-degradation (Albrechtsen *et al.*, 2013). As ADAM10 had been shown to promote chick NCC emigration by cleaving and processing N-Cadherin (Shoval *et al.*, 2007), it remains to be examined whether ADAM10 regulates MMP16 in NCCs, as part of its triggering the onset of NCC migration.

N-Cadherin is down-regulated during NCC EMT (Taneyhill and Schiffmacher, 2013). This substrate was found to be modified by MT-MMPs in various contexts (Folgueras *et al.*, 2009; Itoh, 2015; Porlan *et al.*, 2014). Our results showed that excess MMP16 leads to its reduced expression in the dNT. Moreover, exposure to MMP16 for only three hours was sufficient to induce extensive loss of N-cadherin in NCC explants. While this effect is rapid, whether N-Cadherin is a direct or indirect proteolytic substrate of MMP16 cannot be concluded. Yet, this finding suggests that the effect of MMP16 on this target is prior to, and not a consequence of, their EMT, since the cells devoid of N-cadherin were still connected and displayed epithelioid, rather than mesenchymal, shape. Also, the *in-vivo* injections of MMP16 caused a restricted reduction of N-Cadherin only at the dNT and not in its more ventral parts or in the adjacent mesoderm. This data raises the possibility of an indirect effect of MMP16 on this substrate, which may be mediated by an MMP16-downstream effector that is exclusively present in/around NCCs. Such an effector may be another proMMP, like MMP2 or MMP9, which is cleaved by MMP16 and in turn degrades N-cadherin. Additional MMP16 target may be ADAM10, which was found to be cleaved by MT-MMPs and to degrade N-cadherin in migrating NCCs (Shoval *et al.*, 2007; Wong *et al.*, 2012). Alternatively, the localized MMP16 effect may be attributed to the presence of endogenous MT-MMP inhibitors such as Reck (Prendergast *et al.*, 2012) or of mediators of MMP16 activation such as TIMPs (Zhao *et al.*, 2004). Whether such effectors are excluded from, or localized at, the dNT, respectively, thus restricting MMP16 proteolytic activity to NCCs, remains to be investigated.

Laminin is a main component of the basement membrane around many embryonic tissues (Duband and Thiery, 1987; Goody and Henry, 2010; Tzu and Marinkovich, 2008), and also found to be a target for MMP16 (Shimada *et al.*, 1999). Interestingly, laminin degradation differs in different cancer cell-lines that were exposed to MMP2 or MMP14, resulting in dissimilar motility properties of those cells (Koshikawa *et al.*, 2000). It is well known that during the time when NCCs detach from the dNT, the basal lamina is degraded (Barembaum and Bronner-Fraser, 2005; Le-Douarin and Kalcheim, 1999; Perris and Perissinotto, 2000). Here we demonstrate that *in-vivo* addition of MMP16 caused a widening of the laminin-lacking gap in the dNT. Yet, as we also previously found that MMP9 is necessary and sufficient for laminin degradation during

NCC migration (Monsonego-Ornan *et al.*, 2012), further studies are required to uncover whether laminin is a direct or non-direct substrate of MMP16 in NCCs.

Materials and Methods

Embryos

Fertile Lohman chick eggs were incubated at 38°C until reaching the desired stages. For *in-vivo* procedures, eggs were windowed and embryos visualized by injecting black ink below the blastodisc. Following manipulations, embryos were incubated up to the required stages and fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, Israel).

RT-PCR

Total mRNA was purified from whole embryos of 10, 16 and 25 somites, from isolated neural tubes explants before or after overnight culturing, or from NCCs that migrated away from the cultured neural tubes. Reverse transcription (RT) was performed using the RT-for-PCR Kit (Clontech, CA USA). cDNA was amplified by PCR in 25 μl reaction solution containing 2 μl cDNA, 1 unit taq-polymerase, 25 μM of each dNTP and 2μM primers. Primers for cMMP16 were: Forward 5'ATATCGATTTCACTATGATCG-TACTCGCAC-3'; Reverse 5'ATGAGCTCAAACCCTACATCACACCCA-3'. PCR was carried out using the following program: 95ºC/5 minutes followed by 35 cycles of 94ºC/40 seconds, 56ºC/45 seconds and 72ºC/45 seconds.

Explants of neural primordia

Hindbrain regions of 5-8ss embryos were sectioned out of the embryos, and neural primordia containing premigratory NCCs were isolated from surrounding tissues using 25% pancreatin solution (Sigma-Aldrich, Israel) (described in Monsonego-Ornan *et al.*, 2012; Sela-Donenfeld and Kalcheim, 1999). Neural tube explants were placed onto 50 μg/ml fibronectin-coated plates (Sigma-Aldrich, Israel) and incubated with control CHO-S-SFM II media (Gibco BRL, MD USA) or CHO-S-SFM II media containing recombinant MMP16 (0.4 ng/μl, Calbiochem, CA USA) for 16 hours. Explants were fixed in 4% PFA or 100% methanol and processed for immunostaining. In some experiments, explants were incubated in control CHO-S-SFM II for overnight followed by addition of 0.4 ng/μl MMP16 for 3 hours.

In-ovo procedures

MMP16 injection

Control CHO-S-SFM-II media or 4 ng/μl MMP16-added media was injected into the lumen of the cranial/cervical neural tube in embryos of 5-8 somites using a pulled-glass capillary (Monsonego-Ornan *et al.*, 2012). Embryos were injected twice in 3 hour intervals and fixed 2 hours after the second injection.

Morpholino electroporation

Fluorescein-conjugated MMP16 morpholino antisense oligonucleotides (MO), directed against 5'-UTR of avian MMP16 or control FITC-MO sequence (GeneTools, OR USA) were diluted in PBS to a working concentration of 2mM. MO sequences are: MMP16 5'AGTGCGAGTACGATCATAGTGAACT-3'; Control 5'-CCTCTTACCTCAGTTACAATTTATA-3'. MOs were injected to the cranial neural tube of 5-8ss embryos (described in Monsonego-Ornan *et al.*, 2012). Electrodes were placed lateral to each side of the neural tube and the electroporation was performed with a BTX 3000 electroporator (Fisher scientific, USA) using four 45 msec pulses of 16 volts with 300 msec pulse intervals (Sela-Donenfeld *et al.*, 2009; Weisinger *et al.*, 2008). For rescue experiments media containing 4 ng/μl MMP16 or control media were injected 6 hours postelectroporation. Embryos were incubated for a total time of 16 hours before harvesting.

In-situ *hybridization and immunohystochemistry*

In situ hybridization (ISH) on whole-mounted embryos or on paraffin-sections was performed as described before (Sela-Donenfeld and Kalcheim, 1999), using DIG-labeled probes against chick *MMP16* and *FoxD3* mRNAs. Staining was detected using an alkaline phosphatase (AP)-coupled anti-DIG antibody and NBT/BCIP substrate (Roche, Switzerland). Immunostaining on whole-mounted embryos, paraffin sections or neural tube explants was performed by blocking the samples with 1% or 3% BSA/PBS (in sections or whole mounts, respectively) or 5% goat serum (explants) for 1 hour at room temperature. Samples were incubated with the following antibodies at 4° C for 16 hours: mouse-anti HNK-1 (1:500, BD Pharmingen, CA USA), mouse-anti N-cadherin (1:100, 1:250 in section or explants, respectively, Zymed, CA USA), rabbit-anti laminin (1:100, Sigma MO, USA), rabbit-anti phospo-hystone H3 (1:50, Santa Cruz Biotechnology, CA USA), rabbit anti-MMP16 (1:100, Abcam, CA USA). Antigen retrieval was performed for laminin and N-cadherin staining by boiling the sections for 5 min in 100mM Tris pH9.5. Secondary anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-rabbit Alexa 594 and anti-mouse Alexa 594 (all 1:500, Molecular Probes, CA USA) were added at room temperature for 2 hours. Actin filaments were stained by incubating explants with Phalloidin (1:500, Sigma, MO USA) for 1 hour at room temperature followed by washes with PBS/tween (Monsonego-Ornan *et al.*, 2012). TUNEL assay was performed as described before (Darzynkiewicz *et al.*, 2008; Kayam *et al.*, 2013). Cell nuclei were visualized using staining with 4',6'-diamidino-2-phenylindole (DAPI) (1:7500, Vector Laboratories, CA USA) for 20 min at room-temperature.

Data analysis and microscopy

Data quantification and statistical analysis

All quantification were conducted using ImageJ 1.410 software for area-measurement and provided as arbitrary unit values. These values were normalized relatively to control averaged values. In explants, areas of NCC migration were calculated as a mean of ~5 explants per treatment, using the following formula: total area occupied by migrating both 'epithelioid' and mesenchymal NCCs, divided by the total area of the neural tube itself. In transverse sections, areas of $HNK1^+$ staining, or length of N-cadherin $^-$ and laminin $^-$ staining were measured and normalized to control.

The average ratio of all treated versus control samples were tested for significance. Statistical analysis was performed by ANOVA using JMP software (SAS Institute, USA). Comparisons were performed using t-test and were found to be significant.

Microscopy

Embryos were analyzed under SZX17 stereomicroscope (Olympus). Explants and sections were analyzed using Eclipce E400 upright microscope (Nikon) or SZX17 stereomicroscope (Olympus). All Images were taken with DP70 CCD camera (Olympus) and DP controller software.

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