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ADAM13 cleavage of cadherin-11 promotes CNC migration independently of the homophilic binding site

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ABSTRACT

The cranial neural crest (CNC) is a highly motile population of cells that is responsible for forming the face and jaw in all vertebrates and perturbing their migration can lead to craniofacial birth defects. Cell motility requires a dynamic modification of cell–cell and cell-matrix adhesion. In the CNC, cleavage of the cell adhesion molecule cadherin-11 by ADAM13 is essential for cell migration. This cleavage generates a shed extracellular fragment of cadherin-11 (EC1-3) that possesses pro-migratory activity via an unknown mechanism. Cadherin-11 plays an important role in modulating contact inhibition of locomotion (CIL) in the CNC to regulate directional cell migration. Here, we show that while the integral cadherin-11 requires the homophilic binding site to promote CNC migration *in vivo*, the EC1-3 fragment does not. In addition, we show that increased ADAM13 activity or expression of the EC1-3 fragment increases CNC invasiveness *in vitro* and blocks the repulsive CIL response in colliding cells. This activity requires the presence of an intact homophilic binding site on the EC1-3 suggesting that the cleavage fragment may function as a competitive inhibitor of cadherin-11 adhesion in CIL but not to promote cell migration *in vivo*.

1. Introduction

Craniofacial development in all jawed vertebrates begins with the induction and migration of the cranial neural crest (CNC) cells, which are multipotent embryonic cells that later differentiate into the cartilage, bone, and ganglia of the face and head (Becker et al., 2013; LaBonne and Bronner-Fraser, 1999; Le Douarin and Kalcheim, 1999). CNC cells arise at the border of the neural plate and migrate extensive distances ventrally via defined pathways to the frontonasal prominences and pharyngeal arches. The CNC undergoes two phases of migration, starting with collective cell migration in which cell–cell adhesion maintains cohesion among the migrating group of cells, followed by a dispersion into single cell migration to reach their final locations (Alfandari et al., 2003; Alfandari et al., 2010; Sadaghiani and Thiebaud, 1987). Defects during early development that disrupt the proper induction, migration, proliferation or differentiation of CNC cells lead to an array of craniofacial abnormalities at birth. Craniofacial defects are among the most common birth defects, and many of these, including cleft lip and palate, can only be repaired by plastic surgery, while others have no treatment available. Elucidating the mechanisms by which the CNC develops is crucial to understanding how to prevent or even correct these craniofacial defects before birth.

In *Xenopus*, the mesenchymal cadherin-11 is an essential protein in the CNC and its levels have to be tightly regulated for proper migration. A decrease or increase of cadherin-11 protein levels caused by translation blocking morpholino oligonucleotides (MO), or overexpression leads to defects in migration (Borchers et al., 2001; Kashef et al., 2009). Cadherin-11 is a type II classical cadherin that contains five extracellular repeats (EC1-5) with immunoglobulin-like folds separated by flexible linkers, followed by a transmembrane and cytoplasmic domain (Becker et al., 2012). The membrane-distal EC1 possesses highly conserved tryptophans and the QAV motif, which function as the adhesive site for homophilic binding of cadherins on neighboring cells (Boggon et al., 2002; Patel et al., 2006). We have previously shown that ADAM13 cleaves cadherin-11 in the CNC (McCusker et al., 2009). Based on predicted molecular weights, the estimated cleavage site was determined to be between EC3 and EC4 and would therefore eliminate the homophilic binding site from the transmembrane stump. We further showed that the shed extracellular fragment of

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cadherin-11 containing EC1-3 retains biological function. Expression of an artificial cleavage fragment in the CNC could restore migration in cells devoid of ADAMs or overexpressing cadherin-11, suggesting that the EC1-3 fragment could promote CNC cell migration, however the mechanism by which it does so is not yet clear (McCusker et al., 2009).

Although maintenance of cell–cell adhesion is not a function that has been clearly demonstrated for cadherin-11, several pieces of evidence point towards this role. Namely, the overexpression of a cadherin-11 variant lacking 210 amino acids from the first two EC domains containing the homophilic binding site promotes CNC migration (Borchers et al., 2001), while replacing cadherin-11 with a mutant lacking the homophilic binding motif prevents CNC migration (Becker et al., 2013; Borchers et al., 2001). These variants are thought to act as dominant negatives of cell–cell adhesion. Cadherin-11 also regulates protrusion formation through its cytoplasmic domain via an interaction with the guanine nucleotide exchange factor GEF-Trio, as well as cell proliferation through binding to β-catenin (Borchers et al., 2001; Kashef et al., 2009; Koehler et al., 2013; McCusker et al., 2009). We have also recently shown that cadherin-11 plays a role in mediating cell–cell contacts during contact inhibition of locomotion (CIL; Becker et al., 2013), a mechanism that is important for the directional migration of CNC cells (Carmona-Fontaine et al., 2008). CIL is defined as the "the stopping of the continual locomotion of a cell in the same direction after collision with another cell" (Abercrombie, 1970). During CIL, cell–cell contacts are initiated by cadherins, which then modify RhoA/Rac1 activity through the non-canonical Wnt/PCP pathway (Becker et al., 2013; Carmona-Fontaine et al., 2008; Theveneau et al., 2010). This in turn affects the polarization of the cytoskeleton and leads to a change of migration direction. The process allows collectively migrating cells to define a cell-free edge at which most of the protrusive activity is concentrated, which allows the leading cells to persistently migrate ventrally away from the group and into their designated pathways. Importantly, loss of cadherin-11 in the CNC or overexpression of the homophilic binding-deficient variant causes defects in CIL (Becker et al., 2013; Kashef et al., 2009).

In this study, we demonstrate that homophilic binding is essential to the function of cadherin-11 during CNC migration. We also observe that a non-cleavable variant of cadherin-11 (Cad11-egf) cannot promote CNC migration *in vivo*, confirming that cleavage is essential for migration. We show that ADAM13 cleavage of cadherin-11 decreases cadherin-11-mediated cell–cell adhesion during CIL *in vitro*. In addition, our results reveal that the shed extracellular domain does not require homophilic binding to stimulate CNC migration *in vivo* indicating a novel function for EC1-3.

2. Materials and methods

2.1. Morpholinos and DNA constructs

ADAM13 morpholino antisense oligonucleotide (MO13) and morpholino against cadherin-11 (MO11) were designed as previously characterized (Kashef et al., 2009; McCusker et al., 2009) and purchased from Gene Tools, LLC (Philomath, OR, USA). Full-length ADAM13 (A13), protease-dead ADAM13-E/A, full-length cadherin-11 (C11), EC1-3-myc, GAP43-mcherry and GAP43-GFP were published previously (Alfandari et al., 2001; Kashef et al., 2009; McCusker et al., 2009). The 5′ untranslated region of cadherin-11 that is recognized by MO11 is not present in the cadherin-11 construct in pCS2+. In addition, four silent mutations were made downstream of the ATG to further prevent binding of MO11. C11-egf was engineered by introduction of a SacI site in the C11 construct immediately upstream of the transmembrane domain (QuikChange) and inserting the EGF-like domain of Xenopus ADAM13 (51 amino acids), amplified with the primers 5′-CTGAACCCCAATCCCTTAACTGTGTTTCTAAATGTAATGG-3′ and 5′-GCTCCAGTACTGAGTCCAGCAGTGACACCTACAGGGAGGT. A13-egf (containing two copies of the EGF-like domain) was made by inserting an AscI followed by a SacI site immediately upstream of the transmembrane domain, and then inserting the amplified EGF-like domain of ADAM13 into the AscI and SacI sites using the primers 5′-AGGCGCGCCTTGTGTTTCTAAATGTAATGG-3′ and 5′-CGAGCTCAGTGACACCTACAGGGAGGT-3′. The non-adhesive cadherin-11 (na-C11) and EC1-3 (na-EC1-3) each contain point mutations at W55A and W57A (W2 and W4 after prodomain removal), and A130M of the QAV motif to disrupt the homophilic binding site as previously reported (Tamura et al., 1998). EC1 and EC1-2 were made by introducing stop codons immediately upstream of EC2 (after F159) with the primers 5′-CCCGGAGTTCTAATTGCATGAAAACTACCACGCAAATGTG-3′ and 5′-TTTCATGCAATTAGAACTCCGGGGGATTATCATTTATGTC, or upstream of EC3 (after F268 with primers 5′-ACCAAAGTTTTAACCACAAAGTGCGTACCCCATGTCTGTG-3′ and 5′-CACTTTGTGGTTAAAACTTTGGTGGATTGTCATTGACATC-3′, respectively.

2.2. Cell culture and protein detection

Cos-7 cells (ATCC) were transfected according to manufacturer's instructions (FuGENE HD, Roche). After 48 h total cellular proteins were extracted with 1X TBS, 1% Triton X-100, 5 mM EDTA, 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Glycoproteins were purified from the cell extract using agarose bound Concavilin A (ConA) beads (Vector Labs) overnight and eluted in reducing Laemmli. Cadherin-11 was detected by Western blot using the mouse monoclonal antibody 1B4 (McCusker et al., 2009), and ADAM13 using the rabbit polyclonal antibody 6615 F (Alfandari et al., 1997). The monoclonal antibody 4F12 was produced against a bacterial fusion protein corresponding to the cadherin-11 EC1-3.

2.3. Embryo manipulation

Handling of *Xenopus laevis* embryos and CNC explants were performed as described previously (Kashef et al., 2009). All constructs were transcribed *in vitro* into mRNA according to manufacturer's description (Ambion Inc.). *In vivo* CNC migration assays by targeted injection of a fluorescent lineage tracer were performed as previously published (Abbruzzese et al., 2014; Abbruzzese et al., 2015; Cousin et al., 2011; Cousin et al., 2012). Eight-cell stage embryos were injected into the D1 blastomere with 333 pg of RFP-flag mRNA, plus 333 pg of all cadherin-11 constructs for overexpression assays. In knockdown experiments, 200 pg of RFP-flag mRNA and 5 ng of MO11 was used alone or together with 80 pg of cadherin-11 constructs. All embryos were raised at 15 °C until they reached tailbud stage and were scored for CNC migration by the presence of RFP-labeled cells in the migration pathways. Embryos were imaged using a Nikon fluorescent dissecting microscope or a Zeiss Stereo Lumar fluorescent stereoscope. For the confrontation and collision assays, 1 ng of mRNA or 2 ng of MO13 was injected into the D1 blastomere of eight-cell stage embryos.

2.4. Confrontation and collision assays

CIL assays were performed as described (Becker et al., 2013; Carmona-Fontaine et al., 2008). For the collision assay, CNC cells were dissociated for three minutes with 0.3 mM EGTA in Danilchiks buffer (lacking $CaCl_2$) as described previously (Becker et al., 2013). Live cell images were taken with Axio Observer.Z1 spinning disc confocal microscope with 10x plan apochromate NA 0.45 air objective using AxioVision 4.8.2 software (Zeiss, Jena).

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