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E-cadherin is required for cranial neural crest migration in *Xenopus laevis*

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ABSTRACT

The cranial neural crest (CNC) is a highly motile and multipotent embryonic cell population, which migrates directionally on defined routes throughout the embryo, contributing to facial structures including cartilage, bone and ganglia. Cadherin-mediated cell–cell adhesion is known to play a crucial role in the directional migration of CNC cells. However, migrating CNC co-express different cadherin subtypes, and their individual roles have yet to be fully explored. In previous studies, the expression of individual cadherin subtypes has been analysed using different methods with varying sensitivities, preventing the direct comparison of expression levels. Here, we provide the first comprehensive and comparative analysis of the expression of six cadherin superfamily members during different phases of CNC cell migration in *Xenopus*. By applying a quantitative RT-qPCR approach, we can determine the copy number and abundance of each expressed cadherin through different phases of CNC migration. Using this approach, we show for the first time expression of E-cadherin and XB/C-cadherin in CNC cells, adding them as two new members of cadherins co-expressed during CNC migration. Cadherin co-expression during CNC migration in *Xenopus*, in particular the constant expression of E-cadherin, contradicts the classical epithelial-mesenchymal transition (EMT) model postulating a switch in cadherin expression. Loss-of-function experiments further show that E-cadherin is required for proper CNC cell migration *in vivo* and also for cell protrusion formation *in vitro*. Knockdown of E-cadherin is not rescued by co-injection of other classical cadherins, pointing to a specific function of E-cadherin in mediating CNC cell migration. Finally, through reconstitution experiments with different E-cadherin deletion mutants in E-cadherin morphant embryos, we demonstrate that the extracellular domain, but not the cytoplasmic domain, of E-cadherin is sufficient to rescue CNC cell migration *in vivo*.

1. Introduction

The cranial neural crest (CNC) is a highly motile and transient embryonic “stem-cell” population specific for vertebrates. The CNC originates at the border between the neural plate and the epidermis, and later differentiates into various cell types of the face and head including cartilage, bone and ganglia (Kalcheim and Le Douarin, 1986; Wagner, 1990). In *Xenopus*, CNC cells have been described as a pseudo-epithelial cell population that emigrate shortly before neural tube closure and migrate ventrally on defined routes into the branchial arches. During this process, CNC cells undergo two phases of migration, moving first collectively as a cohesive sheet, which then progressively dissociates into small cell clusters and individual cells (Alfandari et al., 2003, 2010). Thus, cell–cell adhesion has to be precisely and continuously modulated during CNC cell migration. Cell–cell adhesion derives its importance not only from ensuring the integrity of the migrating CNC sheet, but also from regulating directional migration of single cells through “contact inhibition of locomotion” (CIL, Carmona-Fontaine et al., 2008). CNC cells express different cadherins, Ca^{2+}-dependent glycoproteins known to promote homotypic cell–cell adhesion. Apart from establishing epithelial cell polarization and the formation of robust cell–cell contacts, cadherins mediate a number of intracellular signalling cascades, modulate cell cortex tension, and promote migration of other cell types (Becker et al., 2012; Maitre et al., 2012; Takeichi, 1995). Importantly, during CNC migration the expression level of different cadherin subtypes varies (Nakagawa and Takeichi, 1998), which could be function related. For example, during CNC emigration in chicken, a switch of

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cadherin expression occurs from N-cadherin and cadherin-6B to cadherin-7 (Nakagawa and Takeichi, 1998; Sauka-Spengler and Bronner-Fraser, 2008). In addition, it was recently shown that E-cadherin is expressed in emigrating avian CNC cells, indicating that it may have a previously unrecognized function in CNC cell migration (Dady et al., 2012; Rogers et al., 2013).

Xenopus CNC cells express several cadherin subtypes, including Cadherin-11, N-cadherin, PCNS and PAPC (Becker et al., 2012; Schneider et al., 2014). Cadherin-11 is a classical type II cadherin and is expressed at the onset of CNC cell migration (Hadeball et al., 1998). Loss-of-function experiments demonstrate that Cadherin-11 initiates filopodia and lamellipodia formation *in vivo*, which is required for CNC cells to migrate into the branchial arches. Cadherin-11 regulates CNC protrusive activity by binding to GEF-Trio via its cytoplasmic domain, modulating small Rho GTPases (Kashef et al., 2009). In addition, Cadherin-11-mediated cell–cell adhesion is important for proper CNC cell migration, since depletion of its adhesive function results in higher invasiveness of CNC due to loss of CIL and directionality (Becker et al., 2013). Furthermore, Cadherin-11 regulates CNC proliferation through binding to β-catenin (Koehler et al., 2013).

Recently, a model of CNC cell migration has been proposed in which N-cadherin mediated cell–cell adhesion leads to cell protrusion instability within a migrating cohesive CNC cluster. FRET analysis revealed N-cadherin-dependent inhibition of Rac1 at contact sites (Theveneau et al., 2010). Such contact-dependent cell polarity regulation allows for collective chemotaxis in presence of a chemotactic SDF-1 signal (Theveneau et al., 2010). Furthermore, it was demonstrated that Wnt/PCP (planar cell polarity) and N-cadherin signalling are important to promote directional CNC cell migration in a mechanism called “chase-and-run”: CNC cells chase placode cells, which express SDF-1. After contact with CNC cells, placodal cells migrate further ventrally invoking CIL (Theveneau et al., 2013).

The protocadherin PCNS (protocadherin in neural crest and somites) is also expressed in CNC cells. When overexpressed *in vitro*, PCNS localizes at cell–cell contacts, in intracellular vesicles and within cell protrusions (Becker et al., 2012; Rangarajan et al., 2006). Knockdown of PCNS leads to disturbed CNC cell migration, round cell shapes and cell sheet disaggregation on fibronectin substrates (Rangarajan et al., 2006). Interestingly, CNC cell migration defects in PCNS morphant embryos can be rescued by co-injection of another protocadherin family member PAPC (paraxial protocadherin). PAPC shares 65% amino acid identity with PCNS and is also expressed in CNC cells, indicating that both protocadherins can promote CNC cell migration *in vivo* (Schneider et al., 2014).

Different cadherin subtypes are thus expressed during CNC cell migration, but the molecular mechanisms by which they regulate directional CNC cell migration are only incompletely understood. Therefore, there is a need (1) to decipher the expression profile of cadherins during different phases of CNC cell migration and (2) to elucidate the molecular mechanisms by which specific cadherin-based cell–cell contacts affect either stable or transient cell–cell contacts, cell protrusion formation and the mechanical properties of cells.

In this study we present the first quantitative and comparative analysis of mRNA expression of six cadherin superfamily members during different phases of CNC cell migration, providing a novel expression profile for these cadherins in *Xenopus* CNC cells. In contrast to other vertebrate model organisms, we demonstrate here that all investigated *Xenopus* cadherins are continuously co-expressed and up-regulated during CNC migration. This contradicts the classical EMT model, developed through studies in mice and avians, describing switches in the expression levels of various cadherins (Nakagawa and Takeichi, 1998; Sauka-Spengler and Bronner-Fraser, 2008). Furthermore, this study reports E-cadherin expression in *Xenopus* CNC cells for the first time. By knockdown experiments we demonstrate that E-cadherin is required for CNC cell migration *in vivo* and protrusion formation *in vitro*. Moreover, we show that loss of E-cadherin is not rescued by co-injection of either XB-cadherin, Cadherin-11 or N-cadherin, pointing to a specific function of E-cadherin in mediating CNC cell migration. Finally, using reconstitution experiments with different E-cadherin deletion constructs, we demonstrate that the extracellular domain, but not the cytoplasmic domain, of E-cadherin is sufficient to restore CNC cell migration.

2. Results

2.1. Characterization of cadherin expression during Xenopus CNC migration

Xenopus cranial neural crest (CNC) cells initially migrate as a cohesive cell sheet and maintain prominent cell–cell contacts during cell movement. The collective migration mode is subsequently lost when they start to migrate individually towards their target sites (Alfandari et al., 2010). During this process, cadherin mediated cell–cell adhesion has to be constantly and strictly regulated to ensure dynamic organization of the CNC cell population. Multiple cadherin subtypes are expressed in *Xenopus* CNC cells (Kashef et al., 2009; Rangarajan et al., 2006; Theveneau et al., 2010). However, it is not known whether these cadherin subtypes are expressed in similar abundances in CNC cells or how the expressions of these cadherins changes during the entire migration process. Therefore, we performed a quantitative analysis to compare the abundances of cadherin subtypes in CNC cells. To analyse the expression of cadherins at different stages of CNC migration, explants of *Xenopus* CNC were dissected from late neurula stage 17, stage 20 and early tailbud stage 23. Stage 17, 20, and 23 represent the pre-migratory, emigrating and migratory cells, respectively. RNAs were isolated from CNC tissues and used for RT-qPCR analysis (Fig. 1A). To verify that the obtained explants consist of CNC cells, RT-qPCR was performed against different known CNC tissue markers, including the transcription factors *twist*, *slug* (also known as *snail2*) and *snail*. Compared to samples from whole embryos, strong expressions of *twist*, *slug* and *snail* in CNC were observed, confirming the identity of CNC tissue (Fig. S1A–C; Fig. 1B). The expression of *twist* increases constantly from stage 17 to stage 23, while the expression of *slug* declines (Fig. 1B). In contrast, *snail* has a nearly constant expression level (Fig. 1B). In order to exclude contamination from adjacent tissues, the samples were tested for the expression of the mesodermal marker *bra* (Smith et al., 1991), the placodal marker *eya1* (David et al., 2001) and the ectodermal marker *K81* (Type I cytoskeletal) (Jamrich et al., 1987) in RT-qPCR. As shown in Fig. 1B, *bra*, *eya1* and *K81* were detected at extremely low levels compared to CNC markers. For example, at stage 17, *bra* expression roughly equals 0.1% of *snail* expression in the CNC sample. Compared to whole embryo samples, *bra* expression in CNC is about 10% at stage 17 and 1% at stage 23 (Fig. S1D), indicating minimal *bra* expression in the explants. The detected levels of *eya1* in the explants are very low in comparison to CNC markers. *Eya1* expression matches about 1% of the expression of *snail* at stage 17 (Fig. 1B). To further verify that explanted CNC did not contain placodal tissue, CNC dissected embryos were left to heal and stained for *eya1* in whole amount *in situ* hybridization (ISH). We observed unimpaired expression of *eya1* on the CNC-dissected side, indicating that the explanted cells do not contain placodal tissue (Fig. S1E). Finally, we dissected CNC and performed ISH with *PCNS* (as a CNC marker) and *eya1* as probes. Isolated CNC displayed strong staining for *PCNS*, but not for *eya1* (Fig. S1F).