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A catenin-dependent balance between N-cadherin and E-cadherin controls neuroectodermal cell fate choices

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ABSTRACT

Characterizing endogenous protein expression, interaction and function, this study identifies *in vivo* interactions and competitive balance between N-cadherin and E-cadherin in developing avian (*Gallus gallus*) neural and neural crest cells. Numerous cadherin proteins, including neural cadherin (Ncad) and epithelial cadherin (Ecad), are expressed in the developing neural plate as well as in neural crest cells as they delaminate from the newly closed neural tube. To clarify independent or coordinate function during development, we examined their expression in the cranial region. The results revealed surprising overlap and distinct localization of Ecad and Ncad in the neural tube. Using a proximity ligation assay and co-immunoprecipitation, we found that Ncad and Ecad formed heterotypic complexes in the developing neural tube, and that modulation of Ncad levels led to reciprocal gain or reduction of Ecad protein, which then alters ectodermal cell fate. Here, we demonstrate that the balance of Ecad and Ncad is dependent upon the availability of β-catenin proteins, and that alteration of either classical cadherin modifies the proportions of the neural crest and neuroectodermal cells that are specified.

1. Introduction

Cadherin proteins are homophilic cell-cell adhesion molecules important for epithelial integrity and whose changes in expression are linked to the epithelial to mesenchymal transition (EMT) during embryonic development (Schafer et al., 2014; Kerosuo and Bronner-Fraser, 2012) and cancer metastasis (Kang and Massague, 2004; Thiery et al., 2009). Neural cadherin (Ncad) and epithelial cadherin (Ecad) are the archetypical type I cadherins that function in a calcium-dependent manner, have five extracellular domains, a transmembrane domain and bind to intracellular components such as α-, δ- (p120) and β-catenin to link to the actin cytoskeleton and intracellular signaling pathways (Koch et al., 1999). Although cadherins have been well studied in cancer cell lines and amphibian tissues (Gheldof and Berx, 2013; Kashef et al., 2009; Scarpa et al., 2015), far less is known about their *in vivo* roles during ectodermal cell fate specification in amniotes.

Ectodermal cells respond to instructive signals early in development to form neural tissue (Gaur et al., 2016; Lamb et al., 1993; Rogers et al., 2008), non-neural ectoderm (NNE), epidermal/placodal tissue (Schlosser, 2014; Nordin and LaBonne, 2014), or neural crest (NC) tissue (Mayor et al., 1995; Selleck and Bronner-Fraser, 2000). The epigenetic and molecular specification of each of these tissues is followed by morphogenetic events such as neural tube closure (NTC) and the epithelial to mesenchymal transition (EMT) of NC cells. Recent studies in chick, amphibian and mouse embryos have identified transcription factors that regulate ectodermal derivative fate specification (Buitrago-Delgado et al., 2015; Bouzas et al., 2016; Macri et al., 2016; Riddiford and Schlosser, 2016; Acloque et al., 2017; Simoes-Costa et al., 2015), NTC (Ray and Niswander, 2016a; Ray and Niswander, 2016b) and NC EMT (Rogers et al., 2013; Schiffmacher et al., 2014; Strobl-Mazzulla and Bronner, 2012). Comparative analysis of early transcriptional regulators shows that many have overlapping expression and directly or indirectly regulate the expression of specific adhesion molecules to control these processes (Ray and Niswander, 2016a; Fairchild et al., 2014; Fairchild and Gammill, 2013; Strobl-Mazzulla and Bronner, 2012). Many transcription factors expressed in early development regulate the expression of cell adhesion molecules, specifically cadherin proteins, and altering cadherin protein expression or function via perturbation of their upstream regulators leads to abnormal embryonic development (Tien et al., 2015; Rogers et al., 2013; Matsumata et al., 2005; Lin et al., 2016). The question remains however, whether the cadherin proteins have independent functions during ectodermal fate specification in addition to their roles in regulating cell movement.

To resolve these issues, we characterize and compare the expression and localization of two cadherin proteins during the separation of

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neural ectoderm, NNE and NC cells in avian embryos. Here, we show that perturbation of Ncad protein directly alters the expression of proteins thought previously as upstream transcriptional regulators, and leads to defects in ectodermal-derivative specification and NC migration. Our results suggest that there are molecular steps downstream of cadherin proteins that regulate fate specification leading to migration defects. The results show that Ecad protein is expressed in the early epiblast, and its expression is maintained in all three ectodermal derivatives, while Ncad protein appears limited to the neural plate and neural tube region and is absent from premigratory and most migrating NC cells, though expressed at high levels in the neural tube, notochord and cranial mesenchyme. Given the overlap of different cadherins in some tissues, we examined their ability to interact *in vivo* and report that in addition to homotypic interactions, Ncad and Ecad can form heterotypic complexes with each other in the neural tube. In addition, altering levels of Ncad leads to compensation in the levels of Ecad in a β-catenin protein dependent manner, and leads to defects in the proper specification of neural, NNE and NC tissues.

2. Experimental procedures

2.1. Embryos

Fertilized chicken eggs were obtained from local commercial sources (McIntyre Farms, San Diego, CA, AA Farms, CA, and Sunstate Ranch, CA) and incubated at 37 °C to the desired stages according to the criteria of Hamburger and Hamilton (HH). All use of embryos was approved by the California State University Northridge IACUC protocol: 1516-012a, c.

2.2. Electroporation of antisense morpholinos and vectors

A translation blocking antisense fluorescein -labeled morpholino to Ncad (NcadMO) was designed (5′-GCGTTCCCGCTATCCGGCACAT GGA-3′), as well as a non-specific control morpholino (ContMO) (5′-CCTCTTACCTCAGTTACAATTTATA-3′). Injections of the fluorescein-tagged morpholinos (0.75–1 mM plus 0.5–1.5 mg/ml of PCI carrier plasmid DNA; as in Voiculescu et al., 2008) were performed by air pressure using a glass micropipette targeted to the presumptive neural plate region at HH stages 4–5. DNA plasmids pCS2-Ncad-GFP (Ncad-GFP)(Shiau and Bronner-Fraser, 2009), Ncad-YFP-Δp120 (AAA-Ncad-YFP) (Chen et al., 2003), and truncated mouse β-catenin (β-cateninΔ90) (Wrobel et al., 2007) plasmids were used (1 mg/ml) and were introduced in a similar manner to morpholinos described above. HH stage 4–5 electroporations were conducted on whole chick embryo explants placed ventral side up on filter paper rings. The Ncad morpholino and vectors were injected on the right side of the embryo and where indicated, controls were injected on the left side of the same embryo. Platinum electrodes were placed vertically across the chick embryos and electroporated with five pulses of 6.3–6.8 V in 50 ms at 100-ms intervals.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) for Pax7 (Developmental Studies Hybridoma Bank (DSHB), Pax7), Ncad (DSHB, MNCD2; DSHB, 6B3; Abcam, ab18203), Cad6B (DSHB, CCD6B-1), Ecad (BD Transduction Laboratories, 610,181; DSHB, 8C2 (Choi and Gumbiner, 1989); DSHB, 7D6 (Gallin et al., 1983)), β-catenin (Abcam, ab6301), and p120-catenin (Cell Signaling, 4989S) was performed as follows: Embryos were fixed in 4% paraformaldehyde made in phosphate buffer for 15–40 min at room temperature. All washes were performed in TBST + Ca2 + + with 0.5% Triton X-100. Blocking was performed with 10% donkey serum in the same buffer. The primary antibodies (1:5–1:10 for all hybridoma antibodies and 1:200–1:1000 for all others, see Table 1 or Key Resources Table) were incubated in the TBST buffer from overnight

Table 1
Antibodies used in study.

Name	Isotype	Species	Dilution	Immunogen	Binding region	Source
ECAD antibodies						
7D6 (Lcam)	Mouse IgG1	Chicken	1:10	Ft1 fragment	N-terminus (Extracellular)	DSHB
610181 (Anti-E-cadherin)	Mouse IgG2a	Human	1:1000	AA 735–883	C-terminus (Cytoplasmic)	BD Transduction Laboratories
07–697 (Anti-E-cadherin)	Rabbit IgG	Human	1:1000	AA 859–874	C-terminus (Cytoplasmic)	EMD Millipore
8C2 (Cadherin, E)	Mouse IgG1	*Xenopus*	1:10	Tryptic fragment	N-terminus (Extracellular)	DSHB
NCAD antibodies						
MNCD2 (Anti-N-cadherin)	Rat IgG2a	Mouse	1:10	AA 308–597	N-terminus (Extracellular)	DSHB
6B3 (Cadherin, N)	Mouse IgG1	Chicken	1:10	Ncad-α-catenin complex	N-terminus (Extracellular)	DSHB
ab18203 (Anti-N-cadherin)	Rabbit IgG	Human	1:1000	AA 800–900	C-terminus (Cytoplasmic)	Abcam
CAD7 antibodies						
CCD7–1 (Cadherin-7)	Mouse IgG1	Chicken	1:10	N-term 597 AA and the human immunoglobulin Fc region.	N-terminus (Extracellular)	DSHB
ab71412 (Anti-Cadherin 7 antibody - N-terminal)	Rabbit IgG	Human	1:50	N-terminal region of human Cadherin 7 conjugated to KLH	N-terminus (Extracellular)	Abcam
CAD6B antibodies						
ab64917 (Anti-K)	Rabbit IgG	Human	1:50	C-terminal region of human K Cadherin	C-terminus (Cytoplasmic)	Abcam
CCD6B-1 (Cadherin-6B)	Mouse IgG1	Chicken	1:10	N-term 605 AA and the human immunoglobulin Fc region.	N-terminus (Extracellular)	DSHB
Catenin complex antibodies						
ab6301 (Anti-beta catenin)	Mouse IgG1	Chicken	1:500	Recombinant full length protein	N/A	Abcam
4989S (Catenin δ-1 antibody)	Rabbit IgG	Human	1:200	Synthetic peptide	N/A	Cell Signaling Technology