

Expression of Cadherin-11 during Organogenesis in the Chick Embryo

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Abstract

Cadherin-11 (cad-11) is primarily a mesenchymal cadherin that appears in delaminating neural crest cells. Its expression correlates with morphogenetic events and the pattern has been studied in mouse, rat and *Xenopus* embryos, but not during avian organogenesis. Our purpose was to investigate this pattern in the chick embryo during organogenesis using immunolocalization and in situ hybridization. Cad-11 was expressed in mesenchyme around the pharynx and aortic arches, eyes, auditory vesicles, lung buds, stomach, and nasal placodes. Neural expression included some cranial ganglia and also new neuroepithelium within the tail bud region undergoing secondary neurulation. We also found expression in epithelia of the developing circulatory and digestive organs. The limb buds, pineal rudiment and mesonephros were also positive. Cad-11 expression became more widespread with development. Our findings support the role of cad-11 as a mesenchymal cadherin, but provide evidence for a wider role that includes epithelial morphogenesis and secondary neurulation.

Keywords: Cadherin, Chick embryo, Organogenesis, Immunolocalization, In situ hybridization, Secondary neurulation

1. Introduction

Although it is found in non-mesenchymal tissues, Cad-11 is primarily a mesenchymal (Type II) cadherin. Mesenchymal cells require a mechanism to maintain contact, and the abundance of cad-11 among some of them suggests that it may mediate weak adhesions, allowing them to maintain loose connections. In morphogenetic zones where cells reside in undifferentiated masses, they can migrate without forming strong adhesions to cells

with a different fate. Thus cad-11 could have a role in cell sorting and communication (Takeichi, 1994; Kimura *et al.*, 1995; Ellerington *et al.*, 1996; Shin *et al.*, 2000; Gumbiner *et al.*, 2005). Cad-11 and N-cadherin serve as markers for cells differentiating into osteoblasts, chondroblasts, myoblasts or adipocytes, all of which stem from a common mesenchymal propagator (Grigoriadis *et al.*, 1988; Grigoriadis *et al.*, 1990; Yamaguchi and Khan, 1991; Kawaguchi *et al.*, 1999; Oberlender and Tuan, 1994; Shin *et al.*, 2000; Kii *et al.*, 2004). Studies of cad-11 knockout in mouse embryos have shown that the embryos were viable and appeared normal, but they had a distinct phenotype: slight reduction of calcification, craniofacial changes and an abnormal shape of the long bones (Kawaguchi *et al.*, 1999; Kawaguchi *et al.*, 2001).

Neural crest cells (NCCs) utilize several cadherins for their distinctive adhesive properties. Cad-11 endows these cells with a property important for migration. During the epithelial-to-mesenchymal transition (EMT) in neural tube, they emerge from this epithelium dorsally and then migrate along specific pathways to destinations throughout the developing embryo where they differentiate into a diverse array of tissues. During EMT they must disengage from their junctions with other cells. The junctions are dynamically regulated at the time of neurulation. During closure of the neural tube in the mouse, one of the tight junction proteins, occludin, is downregulated, causing the tight junctions to become non-functional (Aaku-Saraste *et al.*, 1996). When tight junctions thus disconnect, cadherin-based adherens junctions remain, but alter their cadherin-specific composition for NCCs disengaging from the neural tube, at least in *Xenopus*, chicken, mouse and rat. In the chicken embryo, premigratory NCCs express N-cadherin and cadherin-6B, but as EMT occurs and migration begins, Ca^{2+} -related adhesion decreases and those cadherins are downregulated (Revel and Brown, 1976; Newgreen and Gooday, 1985; Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998). In their place cadherins associated with migration and the mesenchymal phenotype, cadherin-7 and cad-11, are upregulated (Tanihara *et al.*, 1994; Hoffman and Balling, 1995; Nakagawa & Takeichi, 1995; Simonneau, 1995; Inoue *et al.*, 1997; Hadeball *et al.*, 1998; Nakagawa and Takeichi, 1998; Vallin *et al.*, 1998; Chalpe *et al.*, 2010).

Kimura *et al.* (1995) have studied the cell binding properties and pattern of expression of cad-11 mRNA in the early mouse embryo. Via whole-mount in situ hybridization they were able to examine embryos up to stage E9.5. More mature specimens could not be studied since hybridization probes do not penetrate large samples. However, they also examined superficial tissues such as limb buds in embryos up to stage E13.5. They reported that the earliest expression of cad-11 was found in the head mesoderm and in mesodermal layers of the tail during the mid-to-late primitive streak stages (Kimura *et al.*, 1995). Cad-11 expression was seen in many regions known to be derived from neural crest cells, such as the mandibular and maxillary processes, and the mesenchymal cells that underlie the ectoderm in the pharynx and the periocular mesenchyme. In the trunk region cad-11 was found to be expressed in somites during formation, appearing initially at the caudal end and spreading through each somite, ending with expression only in the sclerotome and core cells. The lateral plate mesoderm was found to express cad-11 in the tail region. It was not found in the notochord, in the outer ectoderm of the entire body, nor in the early cranial or dorsal root ganglia. Cad-11 expression was found to be most intense in mesenchyme and in tissues or structures derived from neural crest cells. Simonneau *et al.* (1995) also studied cad-11 mRNA expression. They studied expression in the rat from pregastrula to very late embryonic stage. They found cad-11 expression in all NCC-derived mesenchyme and also in other mesenchyme, including early expression in the prechordal and paraxial mesoderm, and in the somite sclerotomes. During organogenesis they found expression in mesenchyme throughout the embryonic body whether NCC-derived or otherwise. They reported strong expression in mesenchyme associated with and condensing near epithelial tissues such as the corneal surface epithelium, optic cup, nasal placode, genital epithelium, lung and kidney. In the limb buds, tail and genitalia they observed proximo-distal and antero-posterior mesenchymal gradient expression. And in the ventricle and outflow regions of the heart, invading mesenchyme within the trabeculae expressed cad-11 strongly. Mesenchymal condensations that eventually differentiate into membranous or endochondral bone, and around blood vessels were positive, but epithelia such as the vessel endothelium, endoderm of the foregut, stomach and intestine, dermamyotome, nasal placode, and lung endothelium, were negative (Simonneau *et al.*, 1995).

This cadherin expression pattern is distinctive and draws attention to the need to investigate the pattern of expression in the chick embryo. Recently, Chalpe *et al.* (2010) reported cad-11 expression in the in migrating neural crest of the avian embryo but this analysis was focused on regulation of expression in that particular cell type. The chick embryo is a longstanding vertebrate model of staged development, needed for comparison purposes, and it remains one of the primary reference models for NCC migration and morphogenesis. In addition, its pattern of primary and secondary neurulation zone overlap more closely resembles the human than does the mouse. We describe here the protein level expression of cad-11 in the chick embryo during organogenesis, with

some confirmation by in situ hybridization showing mRNA expression.

2. Materials and Methods

This study was carried out in laboratories within the Department of Biology at the University of Northern Iowa, and within the Biology Department and Neuroscience Group at the University of South Dakota, between 2006 and 2009. The work described here complied with protocols approved by the University of Northern Iowa Animal Care Committee and adhered to the legal requirements of the United States of America.

2.1 Embryos

Fertile eggs from the crossed strains Black Astralorps and New Hampshire Red chickens were obtained from Sun Ray Chicks Hatchery, Hazelton, IA. Younger embryos were removed by placing a Whatman 3MM filter paper ring over the blastoderm and cutting around it with scissors. Older embryos were removed by placing curved forceps under the embryo and cutting around it. Four embryos were studied at each stage, one as a control with omission of primary antibody. Embryos were rinsed in chick Ringer's saline solution to remove yolk and determine the developmental stage according to the Hamburger and Hamilton (1951) staging series. We examined stages from 13 (about two days of development) to 22 (3.5-4 days of development), with study up to stage 25 by whole mount in situ hybridization.

2.2 Tissue processing, microwave antigen retrieval and immunostaining

Embryos were extracted from eggs at various stages and placed in fixative (4% formaldehyde, freshly prepared from paraformaldehyde, in PBS, pH 7.4) on ice for 30 minutes or more. Fixed embryos were washed twice in 50% ethanol (Et-OH) for 30 minutes at room temperature and then transferred to 70% Et-OH. Extraembryonic membranes were dissected away and embryos were placed in a shallow plastic container filled with 500 ml of 100mM Tris buffer, pH 10. They were then irradiated with microwaves in a Tappan microwave oven (model number 56-9431) twice at 640 watts for five minutes each, alongside two liters of water in separate plastic beakers to absorb excess heat. After cooling to room temperature the embryos were incubated in 0.5% bovine serum albumen, 1% Tween-20, and 1% non-fat dry milk in PBS (blocking solution) for 30 minutes.

To immunostain, the embryos were incubated in mouse monoclonal anti-human cad-11 (0.5 mg/ml, Invitrogen, 32-1700/clone 5B2H5) diluted 1:400, in blocking solution at 5°C overnight. This is a mouse IgG1-kappa antibody directed against recombinant protein derived from an intracellular sequence of human cad-11 (aa 651-796) and has not been found to cross-react with other cadherins (Akins *et al.*, 2007). After binding, the primary antibody was removed and the embryos were washed twice in PBS for 15 minutes each at room temperature. To detect primary antibody binding Alexa Fluor 488 tagged anti-mouse IgG (2 mg/ml, Molecular Probes, Inc.) diluted 1:500 in blocking solution was added to the embryos for 30 minutes at room temperature. Embryos were washed in PBS for 15 minutes and stored in 70% Et-OH until observation.

2.3 Synthesis of probes and in situ hybridization

Embryos were fixed overnight in 4% formaldehyde prepared from paraformaldehyde (pH 8.0) at 4°C, dehydrated in the increasing methanol-PBS + Tween-20 (PBT) series (75% PBT-25% methanol, 50% PBT-50% methanol, 25% PBT-75% methanol and 100% methanol) and stored in 100% methanol at -20°C. Antisense and sense cadherin-11 EC3 (bp 1438-1815) RNA probes were synthesized with UTP-digoxigenin (Roche) label. The probe was detected with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche, used at 1:2000 and diluted in 5% goat serum with Tris buffered saline with 0.1% Tween 20 and 0.5% Triton X 100 (TBSTT) followed by the color reaction with the substrate Nitro-Blue Tetrazolium Chloride (NBT; Fisher Biotech) and 5-Bromo-4-Chloro-3-Indolyl Phosphate p-toluidine salt (BCIP; Fisher Biotech). All probes were used at 1 mg/ml diluted in the pre-hybridization buffer (50% formamide, 5 X SSC-pH 4.5, 50 mg/ml yeast RNA, 1% SDS and 50 mg/ml heparin). The antisense probe (377 bases) was synthesized using SP6 polymerase (Promega) and the sense probe (377 bases) synthesized using T7 polymerase (Promega), the plasmid being linearized with NotI (Promega) and BamHI, respectively. Embryos were embedded in paraffin and sectioned at 18 µm.

2.4 Western blotting

Stage 19 embryos were homogenized in 15 mM Tris, 1 mM EDTA, 1% Triton-X100 with 1X Protease Inhibitor Cocktail (Sigma) and sonicated. Following homogenization the protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA), and the homogenate was mixed with an equal volume of 2X Laemmli sample buffer. The mixture was boiled 5 min and loaded into lanes of a 10% polyacrylamide-SDS gel for electrophoresis and subsequent transfer to PVDF (Bio-Rad) membrane. Following transfer the membrane was pre-incubated in Aqua block (EastCoast Bio) for 1 hr at RT. It was then incubated in monoclonal anti-cad-11 antibody (Invitrogen, 32-1700) 2 µg/mL for 1 hr. Following washing in 1X TBSTT, binding of the primary

antibody was detected using the IRDye 680 nm-labeled goat anti-mouse secondary antibody, (1:5,000, LI-COR) for detection with the Odyssey infrared imaging system (LI-COR). Ladder: Precision Plus Protein Standards (Bio-Rad).

2.5 Photography

Whole mount embryos were placed in a dish on the stage of a Leica MZ16 dissecting microscope (Leica Microsystems, Wetzlar, Germany). Images were captured using an Optronics CCD digital camera (SN BG602210-H) with Magnafire[®] computer software (Optronics Inc., Goleta, CA). Images of embryo sections were captured using a Leica DMIRE2 inverted microscope with FITC filter set (Leica Microsystems, Wetzlar, Germany) using the same camera and software. The in situ hybridization images were taken with the Leica MZ16F stereomicroscope, but using a QICAM 12-bit Color Fast 1394 Cooled camera, Model: QIC-F-CLR-12-C.

2.6 Histology

Embryos were dehydrated in an Et-OH series, cleared in xylene and embedded in paraffin. They were sectioned at 7µm thickness and the sections were transferred onto slides coated with Histogrip (Invitrogen) and allowed to dry. Sections were rehydrated and mounted in 9.6% Mowiol 40-88 (Farbwerke Hoechst, AG) made up in 24% glycerol-0.1M Tris buffer, pH 8.5).

2.7 Analysis

Averages of 450 sections for larger embryos, and 250 sections for smaller embryos, were viewed and analyzed. Based on direct observation tissues were assigned as having negative, low intensity or high intensity staining compared to control tissue sections where the primary antibody was omitted.

3. Results

We studied cad-11 expression in whole chick embryos and sections during Hamburger-Hamilton (1951) stages 13-25. We used a monoclonal antibody directed against a unique intracellular segment of the protein for immunolocalization. A western blot of stage 19 homogenized embryonic chick tissue is shown in Figure 1A. The blot revealed a band recognized by the monoclonal antibody to cad-11, of approximately 115 kDa. We also studied its mRNA expression via in situ hybridization in whole embryos and sections using a riboprobe complementary to cad-11 mRNA. A complete list of tissues analyzed is displayed in Table 1.

3.1 Cad-11 expression viewed in whole mount embryos

The immunolocalization of cad-11 in whole mount embryos revealed positive staining from stage 13 onward. The staining reached high intensity by stage 18 (Figure 1B) in the head mesenchyme, especially near the eyes, nasal pits, and around the telencephalon, but eventually also around the diencephalon and mesencephalon. The pharynx region also stained with high intensity from early stages, including and especially the branchial arches (Figure 1B). The strong staining in the pharynx was apparent posteriorly to include the lung buds, esophagus, stomach, and liver rudiments. Staining in the heart did not appear until stage 18 and was then intense only in the loop of the ventricle. Low intensity staining was easily discernible in the auditory vesicles and semilunar ganglia as they developed in the head, and in a segmental pattern along the body axis that reflected the periodicity of the somites, apparently between them. The limb buds, as they developed, also stained with low intensity in whole embryos, and increased in intensity somewhat with development to stage 22. Based on this increase and on their appearance in sections they were classified as having high intensity staining (Table 1).

The in situ hybridization of whole embryos is shown in Figure 1, D-F and Figure 3, E-G). In early embryos cad-11 mRNA was apparent in the head mesenchyme and pharynx, and segmentally along the body axis. The posterior end of the neural tube was clearly stained though there was no staining anterior to this (Figure 1D). The pattern of cad-11 mRNA expression was the same as that seen in immunolocalization generally, except that by stage 18 the heart appeared to be negative (penetration of riboprobe into this dense tissue may have been limited), and staining was visible in the aortic sack. In addition, the limb buds and periodic staining of intersomitic tissue were relatively more intense (Figure 1E, F). By stage 20 it was possible to clearly see strong staining of the secondary neural tube, and this remained only in the caudal-most area of the tail (shown in Figure 3, D-G). By contrast, the primary neural tube remained negative (Figure 1E and Figure 3, F and G). As the pharyngeal arches developed the expression of cad-11 there became stronger and more defined (Figure 1, D-F).

3.2 Cadherin-11 expression viewed in transverse sections

Immunostaining for cad-11 in cross-sections was analyzed developmentally and organized according to tissue type in the developing organ rudiments of the nervous, circulatory, and digestive systems, and the limb buds. We

describe the expression developmentally within each system and refer to labeled images in Figure 2, which show many but not all tissues. However, images showing cad-11 expression during secondary neurulation are displayed in Figure 3.

3.2.1 Developing nervous system and associated mesenchyme

Components of the nervous system that were positive for cad-11 expression were limited to some ganglia and sense organs. Of the major ganglia in the head only two pairs, the semilunar and jugular were strongly positive. Anti-cad-11 antibody stained the semilunar ganglia with high intensity in stage 17-22 embryos, but the jugular ganglia were initially positive beginning at stage 22. The acoustico-facialis ganglia were positive and showed low intensity staining during these same stages. The epibranchial placodes, which later contribute to the facialis portion of the acoustico-facialis ganglia, however, did stain with high intensity at stages 17-20. In sections containing the superior ganglia and the dorsal root ganglia it was not possible to detect any immunostaining of these tissues.

During the early stages of brain development the mesenchyme surrounding brain segments appeared positive for cad-11. At stage 15 the mesenchyme near the telencephalon became positive and at stage 16 the mesenchyme around the mesencephalon was positive. These areas remained positive at low intensity. The mesenchyme near the diencephalon became positive at low intensity later, beginning between stages 18 and 20. The mesenchyme near the myelencephalon appeared to remain negative. At stage 22 the pineal epithelial rudiment on the dorsal side of the diencephalon stained positive and appeared to stain most intensely at curvatures where cells are wedge-shaped (Figure 2G). The mesenchyme directly overlying the pineal evagination was not positive. However near its edges, where the epithelium of the pineal gland was most positive, the mesenchyme did stain for cad-11.

The auditory vesicles were also positive for cad-11 but staining was mainly localized to the basal and especially the apical ends of the long, narrow cells (Figure 2B). In this epithelium a distinct line of bright staining could be seen near the lumen that resembled a line of adherens junctions. The cornea stained with high intensity although it was variable and stained with low intensity at stages 20 and 21. The lens epithelium and optic cup near it stained with low intensity, but the medial (deeper) aspects of the eye were negative. However, the periocular mesenchyme did stain positive at stages 14-22, the brightest signal appearing near the edges of the optic cup and in a few cells very near the pigmented retina (Figure 2E). The ectodermal epithelium of the nasal placodes, visible by stages 16-17, as well as the mesenchyme adjacent to them, stained positive for cad-11. The somites stained positive at low intensity for cad-11 during early formation. As they became epithelial we observed low intensity expression throughout except for somewhat higher expression in the dermamyotome (Figure 3B). As they matured staining became fainter and eventually disappeared.

Although the primary neural tube was unstained through the length of the embryo at stages 15-22, the small secondary neural tubes that formed and coalesced in the tail bud did transiently stain positive for cad-11 as shown at stage 15-16 (Figure 3B and C) and were positive for cad-11 mRNA as shown at stage 13 (along with the lateral plate mesoderm, Figure 3A) and at stage 20 (Figure 3D).

3.2.2 Cad-11 expression in the developing circulatory system and associated mesenchyme

We observed cad-11 expression in the chick embryo developing circulatory system and found that staining became more widespread in later stages. Sections showing the dorsal mesocardium, through which new cells enter the heart, stained positive for cad-11 consistently, indicating expression in precardiac migrating cells throughout stages 13-22. However, areas of the heart myocardium appeared positive only in the later stages. Beginning at stage 18 the myocardial wall of the ventricle loop was positive although the endocardium in all segments was negative. The ventricle remained positive with low intensity staining at stage 22 (shown at stage 19 in Figure 2C). The conotruncus was positive at stage 21, and the aortic sac stained at stage 22. In agreement with staining seen in whole mount embryos, the heart tissue seen in sections did not stain during the earlier stages of development, but once staining appeared, it gradually increased in extent and intensity with development. Overall, the positive areas of the heart seen in sections stained with low intensity, but the ventricle showing high intensity at the inner curvature of its loop (Figure 2C). The mesenchyme surrounding the aortic arches, derived from neural crest cells, stained with high intensity through all the stages of development studied.

3.2.3 Expression of cad-11 in the digestive tract and associated tissues

The digestive system tissues consistently stained positive for cad-11. The mesenchyme surrounding the pharynx expressed cad-11 through all of the stages examined, especially in the mandibular and maxillary processes (Figure 2G). The endoderm of the pharynx, though negative from stages 13-18, became positive during stage 19

and remained so through stage 22. A similar pattern and sequence was seen in other parts of the digestive tract. The mesenchyme surrounding the laryngotracheal groove, the esophagus and the stomach were identified as positive earlier, from stages 16-22, and then the endoderm of each of these became positive as the embryo developed, appearing at stages 20-22 (Figure 2A and C). Mesenchyme surrounding the lung buds also appeared positive from an early stage (stage 16) and remained positive throughout the period studied. The endothelium of the lung buds became positive during stage 17 (Figure 2A). The mesenchyme of the dorsal mesogaster was positive for cad-11 during stages 17-22, and from the time of its appearance at stage 14 the cranial liver rudiment showed high intensity staining (Figure 2C).

In the urogenital tissues, we observed that the endoderm and mesenchyme of the cloaca stained positively with low intensity for cad-11 during stages 17-22. The pattern was the same for the hindgut. The mesonephric ducts (Figure 3B) and tubules stained positive for cad-11 as soon as they appeared, and with high intensity from stage 16 onward.

3.2.4 Expression of cad-11 in the developing limb buds

The wing and leg buds stained positive for cad-11 from stages 18 through 22. The mesenchyme of the limb bud stained with high intensity (though not as high as some tissues) and fairly uniformly throughout the limb bud (Figure 2F). A taper in intensity from the interior to the exterior edge (brighter), and a slight taper from proximal-to-distal (brighter brighter) of the limb bud was perceptible. But the background fluorescence in unstained limb buds (control sections stained using no primary antibody) showed a similar taper suggesting that the pattern is partly the result of changing cell density through the wing and leg buds or incomplete antibody penetration. The ectodermal covering of the limb buds was negative except for the apical ectodermal ridges (AER), which stained with high intensity for cad-11 during stages 20-22 (Figure 2F). Localization of cad-11 mRNA in stage 22-25 limb buds revealed the entire ectodermal covering as negative, and the mesenchyme as positive, with a more definite proximal-to-distal gradient than seen with immunostaining, especially in the anterior distal region (Figure 1, E and F, and Figure 3, F and G).

4. Discussion

By studying the expression of cad-11 we are better able to understand its functional role in embryonic development. Our results provide evidence that not only supports the known feature of cad-11 as an adhesion molecule in mesenchyme, but also its potential involvement in morphogenetic events such as epithelial rearrangements.

For the developing nervous system, our results show that in the chick embryo some major ganglia in the head express cad-11 whereas the dorsal root ganglia do not. These results differ from the findings of Kimura *et al.* (1995) and Simonneau *et al.* (1995) who did not find cad-11 expression in cranial or dorsal root ganglia for the early stages of development in the mouse or rat. Our observation of cad-11 expression in the semilunar and other cranial ganglia is not surprising, however, since they receive contributions of NCCs, which express cad-11 as they begin to migrate. Kimura *et al.* (1995) also reported mRNA expression in the neural tube. We found expression of cad-11 protein in the primary neural tube only in the posterior end early, at stage 13. However, we found expression of cad-11 during secondary neurulation during stages 13 through 25 (Figure 3, A-G). Expression in tissues undergoing secondary neurulation (Schoenwolf and De Longo, 1980; Saraga-Babic *et al.*, 1996; Yang *et al.*, 2003) is interesting because these cells are going through a mesenchyme-to-epithelial transition, forming a neural tube through the process of cavitation of tailbud mesenchyme rather than elevation, apposition, and fusion of neural folds. This conversion is important for the cells that will help to form the caudal end of the neural tube in a process that follows a schedule of differentiation independent from that of the primary neural tube (Chung *et al.*, 2008). A recent study has established the identity of the cells that originate formation of the secondary neural tube in the caudo-medial region of Hensen's node at stage 8 (Shimokita and Takahashi, 2011). In this region the basal lamina disappears from the epiblast epithelium as cells become mesenchymal, and then they express N-cadherin as they begin to associate again in the tailbud (Shimokita and Takahashi, 2011). Our finding that expression of cad-11 in the cells during the subsequent cavitation process suggests that it is also involved in the organization of those cells. The pattern of these transitions appears to differ from that in the mouse (Nievelstein *et al.*, 1993; Catala *et al.*, 1995). It will be important to examine these early stages for cad-11 expression.

Expression of cad-11 in parts of the circulatory system was evident. Other studies have found cad-11 mRNA expression in vascular smooth muscle cells (Monahan *et al.*, 2007), endocardial cushion (Shelton and Yutzey, 2008) and other mesenchymally derived cells including cells among the ventricular trabeculae (Simonneau *et al.*, 1995), likely NCCs. But our finding that the embryonic heart does express cad-11 in the ventricular wall is

distinctive. We found expression at stage 13 in the dorsal mesocardium where precardiac mesodermal cells enter, and a later progression of expression in the myocardium of the ventricle (Figure 2C), conotruncus, and the aortic sac. We did not see expression in the atrium. Increasingly wider expression in older embryos suggests that cad-11 is upregulated as the heart becomes more complex. Cad-11 was consistently expressed in the pharynx near the aortic arches throughout all stages of development. Much of this expression in the outflow region of the heart and vessels likely reflects the contributions of cardiac NCCs.

The mesenchyme of the pharynx region stained positive for cad-11 throughout all the stages of development that we examined, including mesenchyme within the mandibular and maxillary processes. This is consistent with findings for the mouse by Kimura *et al.* (1995) who found these particular tissues to be the most intensely stained. Similar expression of cad-11 in the branchial arches was found in the rat (Simonneau *et al.*, 1995) and in *Xenopus* (Vallin *et al.*, 1998). Our results, as well as those by Kimura *et al.* (1995), show that expression of cad-11 in this region gradually decreases as the embryos develop. This mesenchyme is contributed by NCCs.

Cad-11 is known as a mainly mesenchymal cadherin and our results for the chick embryo provide much supporting evidence. The periocular, corneal, and auditory vesicle mesenchymes were particularly interesting as these NCC-derived tissues stained at every stage studied except stage 13. Our results support the findings of Kimura *et al.* (1995) in the mouse and Simonneau *et al.* (1995) in the rat. However, we have found that in the chick embryo, cad-11 expression was considerably more intense in the periocular mesenchyme than in the auditory vesicle mesenchyme (compare Figure 2B and E), in contrast with the results of Kimura *et al.* (1995), which show more cad-11 mRNA expression in auditory vesicle mesenchyme. Positive mesenchyme was also observed near the nasal placodes, lung buds and stomach, and in the dorsal mesogaster. The function of cad-11 expression in mesenchyme is likely to be that of allowing cells to maintain a loose connectivity with one another while promoting migration and shape-changing. Its expression was most intense in areas actively undergoing morphogenesis. These masses of undifferentiated cells may use cad-11 during the processes of migration, sorting and communicating with other cells without creating strong adhesions to them.

As in other mesenchymal tissues in the embryo, cad-11 is expressed in the limb bud mesenchyme. The mRNA expression pattern is a distinct gradient of expression increasing from proximal-to-distal with highest expression at the distal tip and within condensing mesenchyme (Figure 3G) and cartilage condensations (Kimura *et al.*, 1995; Simonneau *et al.*, 1995). Our *in situ* hybridization in whole mount embryos (Figure 1E, F) and on sections through limb buds (not shown), confirm the gradient patterns. However, our observations of avian cad-11 protein expression during limb bud development differ somewhat from findings for mRNA in mouse by Kimura *et al.* (1995) and in the rat by Simonneau *et al.* (1995). Through stage 23 we found positive cad-11 expression in the core of the limb where cavitations were appearing, and no readily apparent increasing concentration toward the periphery. In addition, the proximal-to-distal expression gradient is not as distinct at the protein level. Our results for protein also contrast with those for mRNA with regard to the AER, which we observed to be positive, though the limb ectoderm was otherwise negative. Cadherins, including cad-11, are subject to a variety of transcriptional and post-transcriptional regulation (Halbleib & Nelson, 2006; Farina *et al.*, 2009) and these differences between protein and mRNA expression patterns suggest that cad-11 may indeed be differentially regulated at the transcript and protein levels, including the possibility of a regional mechanism of translation or transport control. It is also plausible that the mRNA pattern of expression is quite transient or dynamic, but protein accumulation is more even.

Our results also show cad-11 protein staining in some epithelial tissues. Our results for the optic cup epithelium are in close accord with results for the mouse (Kimura *et al.* 1995) as in both animals the outer region expressed more strongly than the inner. The auditory vesicle did not stain generally for cad-11, however, within some areas there was strong staining at basal and especially apical sides of the epithelium. Kimura *et al.* (1995) also reported cad-11 mRNA expression in optic vesicle and auditory vesicle for the mouse, and Luo *et al.* (2007) have reported expression in supporting cells and homogeneous cells of the developing chick cochlea. Our observations of cad-11 protein expression in the auditory vesicle suggest that it may be involved in the cell-shape-change-driven complex morphogenetic events that form the inner ear. We saw similar patterns for the pineal gland rudiment, lung buds and the stomach. As the auditory layer cells developed into closed auditory vesicles, a distinct line of apical staining became visible, appearing much like a line of adherens junctions. We also detected cad-11 in the endothelium of the pharynx, pineal, laryngotracheal groove, lung buds, esophagus, stomach, and liver as the embryo developed. The epithelium of the mesonephric ducts and tubules stained positive from stages 16-22 as well.

We observed cad-11 expression in newly formed somites, where apical junctional lines, again, could be seen among the cells. Expression was initiated in the somites as they became closed epithelial vesicles, and as they

matured cad-11 staining became faint. Our observations of initial expression of cad-11 in the newly formed epithelial somitic vesicles are consistent with the results reported by Kimura *et al.* (1995) for the mouse embryo. The expression of cad-11 in epithelial cells suggests that it may be involved in branching morphogenesis (Simonneau *et al.*, 1995), perhaps via the signaling events that cause active cell shape changing via activation of the actin cytoskeleton during organogenesis (Watanabe *et al.*, 2010).

Other groups have noted that neural crest cells upregulate cad-11 coincident with migration (Kimura *et al.*, 1995; Simonneau *et al.*, 1995; Hadeball *et al.*, 1998; Vallin *et al.*, 1998; Chalpe *et al.*, 2010). Although we did not detect neural crest cell streams in this study, we did find that many neural crest cell derivatives stained positive for cad-11, suggesting that these cells express cad-11. Failure to detect neural crest cell streams may be due to a level of expression too low to be detected as these cells are delaminating and migrating away from the neural tube, but which later becomes detectable. Messenger RNA localization by in situ hybridization does show that these cells are positive (data not shown), and also positive for protein by immunostaining in explant culture (Chalpe *et al.*, 2010). That study also showed that only some but not all neural crest cells, labeled with HNK- 1 antibody, expressed cad-11.

We have characterized the expression pattern of cad-11 during early stages of chick development. This is the first descriptive study of the expression pattern for chicken. Cad-11 protein expression has a distinctive profile when compared to other cadherins. Unlike most, it is expressed broadly throughout the embryo and mainly in mesenchyme. But we find there is also expression in certain epithelia, particularly those involved in active morphogenesis. By understanding how cad-11 expressing cells interact with other cells, and the properties imparted to those cells (i.e. motility or changing shape), we are able to better understand why cad-11 is expressed where and when it is. Future studies to understand cad-11 function in these tissues should involve blocking expression of cad-11 protein in the chick embryo.

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Table 1. Tissue assessment for cad-11 expression

Negative	Low Intensity	High Intensity
Superior ganglia	Acousticofacialis ganglia	Semilunar ganglia
Infundibulum	Aortic sac	Jugular ganglia
Dorsal root ganglia	Ventricle myocardium	Epibranchial placode
Aorta	Dorsal mesocardium	Corneas
Atrium	Dorsal mesogaster	Aortic arches
Neural tube (primary)	Laryngotracheal groove	Cranial liver rudiment
Oculomotor nerve	Trachea	Mesonephric duct
Facial nerve	Lung buds	Mesonephric tubule
Spinal nerve	Stomach	Auditory vesicles
Tail bud	Somites	AER
	Nasal placodes	Wing buds
	Cloaca	Leg Buds
	Hindgut	Pineal gland
	Mesenchyme near...	Secondary neural tube
	Auditory vesicles	Mesenchyme near
	Nasal placodes	Eye
	Pineal rudiment	Pharynx
	Telencephalon	Aortic arches
	Mesencephalon	Laryngotracheal groove
	Diencephalon	Lung buds
		Stomach
		Dorsal mesogaster
		Esophagus

Immunostaining intensity for cad-11 was categorized for embryonic chick tissues observed during Hamburger-Hamilton stages 13-22 as absent, low intensity, or high intensity.

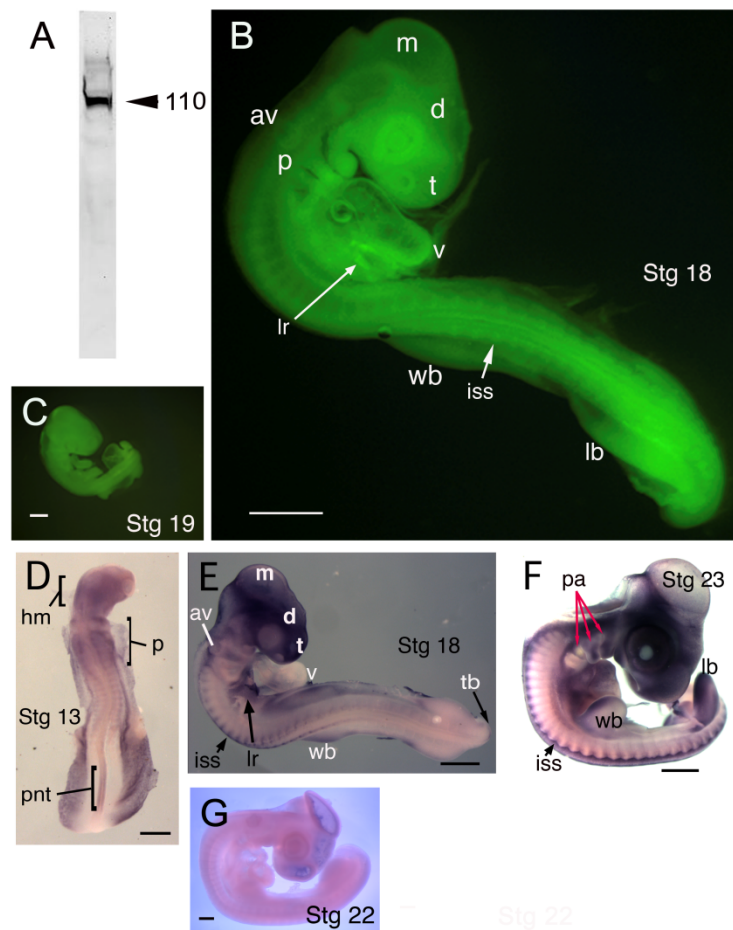


Figure 1. Western blot and localization of cad-11 in whole mount embryos

A: Western blot of stage 19 chick embryo homogenate separated on a 10% polyacrylamide gel, transferred to PVDF membrane, and probed with a monoclonal antibody to cad-11. The antibody bound to a band of molecular weight 100-120 kDa. B: Stage 18 embryo stained with monoclonal anti-cad-11. Bright fluorescent stain is visible in mesenchyme near the diencephalon, telencephalon, and eyes. The auditory vesicles, nasal placodes, ventricular loop, pharynx, and liver rudiment also stained intensely. Some staining of the intersomitic spaces along body axis and the limb buds can be seen as well. Bar = 650 mm. C: An embryo at stage 19 was immunostained with primary antibody incubation omitted and shows only background fluorescence. Bar = 260 mm. D-F: Series of whole mount embryos stages 13-23 showing developmental profile of cad-11 mRNA expression. The embryos were stained by in situ hybridization with an anti-sense probe for cad-11. At stage 13 there was moderate staining of head mesenchyme and pharynx, periodic staining along the body axis, and evident staining of the posterior segment of the primary neural tube. Bar = 500 mm. At stage 18 the staining was generally present in the same areas stained by immunolocalization (seen in B), but the limb buds appeared to show more definitive and tapered expression, the ventricle was less stained, and the periodic trunk staining was recognizable as prominent in the intersomitic spaces. Just visible at the tail's curvature is a confined region of staining near the tailbud. Bar = 650 mm. At stage 23 cad-11 expression became more prominent and resolved in each of the pharyngeal arches, intersomitic spaces, and limb buds. Bar = 400 mm. G: Sense probe control stage 22 embryo. Bar = 260 mm. E: Abbreviations: av, auditory vesicle; d, diencephalon; hm, head mesenchyme; iss, intersomitic space; lb, leg bud; lr, liver rudiment; m, mesencephalon; p, pharynx region; pa, pharyngeal arches; pnt, posterior neural tube; t, telencephalon; tb, tailbud; v, ventricular loop; wb, wing bud.

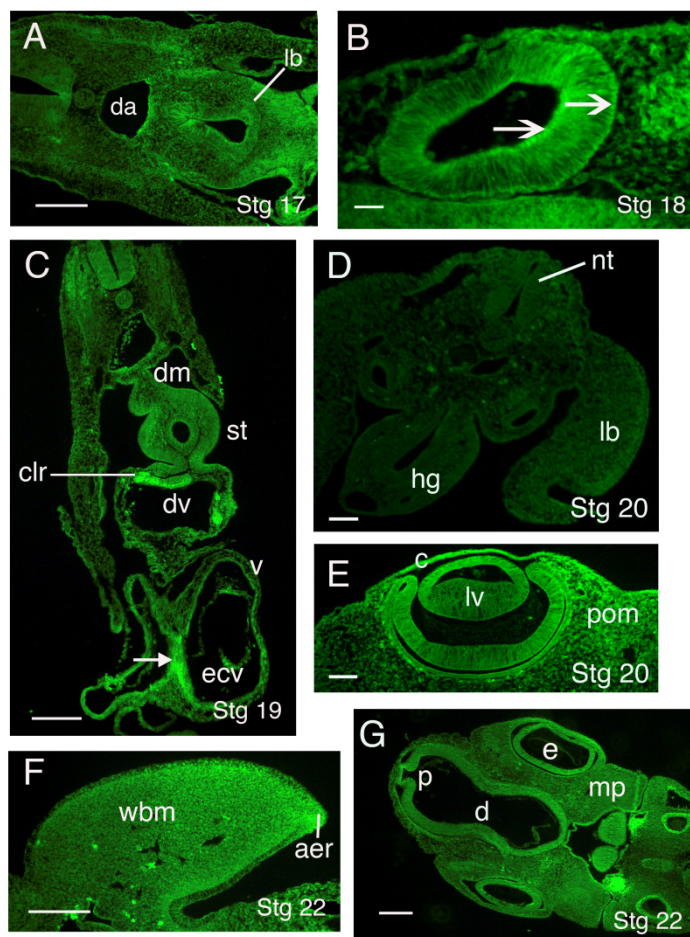


Figure 2. Immunolocalization of cad-11 in sections

A: Section through trunk of stage 17 embryo. The endoderm of the pharynx and lung buds shows strong staining, especially apically, and the surrounding mesenchyme is also positive. Bar = 850 μ m. B: Section through auditory vesicle of stage 18 embryo. High intensity cad-11 staining is evident at apical and basal sides (arrows) of this epithelium. Bar = 100 μ m. C: Section through trunk of stage 19 embryo. The dorsal mesogaster and stomach, both in endothelium and mesenchymal wall, immunostain with low intensity. The cranial liver rudiment stains with high intensity. The ventricular myocardium stains with low intensity, but with high intensity at concave side of the ventricular loop (arrow). Bar = 1000 μ m. D: Immunostaining control stage 20 embryo, section through the trunk. The primary antibody was omitted during immunostaining. Only background fluorescence is visible. Bar = 400 μ m. E: Section through eye of stage 15 embryo immunostained with antibody to cad-11. The cornea and periocular mesenchyme stain intensely. The anterior lens epithelium of the lens vesicle and the edges of the optic cup stain with lower intensity. Bar = 100 μ m. F: Section through wing bud of stage 22 embryo. Cad-11 staining is intense in both mesenchyme and apical ectodermal ridge at this stage. Bar = 10 μ m. G: Section through rostral side of eyes and diencephalon of stage 22 embryo. The pineal rudiment shows high intensity immunostaining near the lumen of the diencephalon. The first pair of aortic arches stain with high intensity within the mandibular arches. Bar = 100 μ m. Abbreviations: aer, apical ectodermal ridge; c, cornea; clr, cranial liver rudiment; d, diencephalon; da, dorsal aorta; dm, dorsal mesogaster; dv, ductus venosus; e, eye; ecv, endocardium of ventricle; hg, hindgut; lv, lens vesicle; lb, lung bud; mp, maxillary process; nt, neural tube; p, pineal rudiment; pom, periocular mesenchyme; st, stomach; v, ventricle; wbm, wing bud mesenchyme.

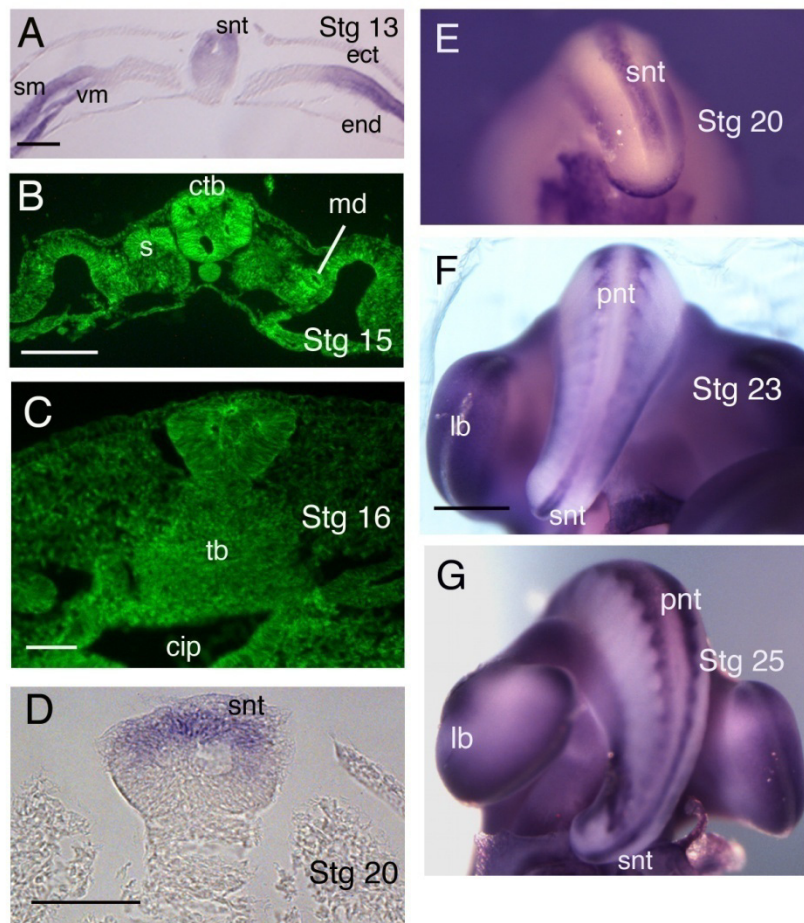


Figure 3. Localization of cad-11 in stages 13-25 tailbud

A: cross-section of stage 13 embryo stained by in situ hybridization. The cross-section was taken through the secondary neural tube in the tail. The dorsal side of the secondary neural tube stains but the segmental plate mesoderm does not. The lateral (extraembryonic) mesoderm shows high levels of cad-11 mRNA. Bar in A = 100µm. B: Section through a stage 15 embryo showing secondary neurulation cavitations with bright cad-11 immunostaining dorsal to the notochord. The dorsal aspect of a somite and the mesonephric duct are also positive for cad-11. Bar = 100µm. C: Section through the tailbud of a stage 16 embryo showing multiple cavitations dorsally. The section is more posterior than the section shown in B. The cells surrounding the small lumina are positive for cad-11. Bar = 50µm. D: Section through the tailbud of a stage 20 embryo at higher magnification stained by in situ hybridization. The larger two fusing cavitations show cad-11 mRNA expression mainly on the dorsal aspect. Bar = 100µm. E-G: Stages 20-25 whole mount embryos stained by in situ hybridization and oriented for a caudal view. The secondary neural tube staining becomes smoother and remains confined to the caudalmost region. The primary neural tube is unstained, however segmental staining at its lateral borders suggests cad-11 positive neural crest cells. Bar = 480µm. Abbreviations: cip, caudal intestinal portal; ctb, cavitating tail bud; ect, ectoderm; end, endoderm; lb, leg bud; md, mesonephric duct; pnt, primary neural tube; s, somite; sm, somatic mesoderm; snt, secondary neural tube; tb, tail bud; vm, visceral mesoderm.