Journal of Cellular Biochemistry 87:342–359 (2002)

Alterations in the Spatiotemporal Expression Pattern and Function of N-Cadherin Inhibit Cellular Condensation and Chondrogenesis of Limb Mesenchymal Cells In Vitro

Anthony M. DeLise[1] and Rocky S. Tuan[1,2]*

[1]Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
[2]Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis and Musculoskeletal & Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract Cartilage formation in the embryonic limb is presaged by a cellular condensation phase that is mediated by both cell–cell and cell–matrix interactions. N-Cadherin, a Ca^{2+}-dependent cell–cell adhesion molecule, is expressed at higher levels in the condensing mesenchyme, followed by down-regulation upon chondrogenic differentiation, strongly suggesting a functional role in the cellular condensation process. To further examine the role of N-cadherin, we have generated expression constructs of wild type and two deletion mutants (extracellular and intracellular) of N-cadherin in the avian replication-competent, RCAS retrovirus, and transfected primary chick limb mesenchymal cell cultures with these constructs. The effects of altered, sustained expression of N-cadherin and its mutant forms on cellular condensation, on the basis of peanut agglutinin (DNA) staining, and chondrogenesis, based on expression of chondrocyte phenotypic markers, were characterized. Cellular condensation was relatively unchanged in cultures overexpressing wild type N-cadherin, compared to controls on all days in culture. However, expression of either of the deletion mutant forms of N-cadherin resulted in decreased condensation, with the extracellular deletion mutant demonstrating the most severe inhibition, suggesting a requirement for N-cadherin mediated cell–cell adhesion and signaling in cellular condensation. Subsequent chondrogenic differentiation was also affected in all cultures overexpressing the N-cadherin constructs, on the basis of metabolic sulfate incorporation, the presence of the cartilage matrix proteins collagen type II and cartilage proteoglycan link protein, and alcian blue staining of the matrix. The characteristics of the cultures suggest that the N-cadherin mutants disrupt proper cellular condensation and subsequent chondrogenesis, while the cultures overexpressing wild type N-cadherin appear to condense normally, but are unable to proceed toward differentiation, possibly due to the prolonged maintenance of increased cell-cell adhesiveness. Thus, spatiotemporally regulated N-cadherin expression and function, at the level of both homotypic binding and linkage to the cytoskeleton, is required for chondrogenesis of limb mesenchymal cells. J. Cell. Biochem. 87: 342–359, 2002. Published 2002 Wiley-Liss, Inc.†

Key words: chondrogenesis; N-cadherin; cell adhesion; condensation; cartilage; limb development; retroviral gene transduction; cell–cell interaction

A hallmark of skeletogenesis is the formation of mesenchymal cellular condensations in the regions destined to become cartilage [Fell, 1925; Fell and Canti, 1935; Thorogood and Hinchliffe, 1975]. The importance of cellular condensation is underscored by the severe skeletal abnormalities accompanying genetic defects that result in defective cellular condensation, such as Brachypod and Phocomelia in mice and talpid in chickens [Gruneberg, 1963; Hall and Miyake, 1992; Mundlos and Olsen, 1997a,b].

Cellular condensations were first described by Fell [1925] who observed that the cells in the chick limb bud destined to become cartilage were more compacted than the surrounding non-chondrogenic cells. Cell condensations in the limb buds form as a result of cellular aggregation towards a center [Hall and Miyake, 2000], resulting in an increase in cell packing

Grant sponsor: NIH; Grant numbers: DE16864, ES07005, AR42887, ES07282, AR45181; Grant sponsor: NIEHS training grant (to A.M.D.); Grant number: T32ES07282.

*Correspondence to: Rocky S. Tuan, PhD, Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, and Musculoskeletal & Skin Diseases, National Institutes of Health, 50 South Drive, Building 50, Room 1503, Bethesda, MD 20892-8022. E-mail: tuanr@mail.nih.gov

Received 1 July 2002; Accepted 29 July 2002

DOI 10.1002/jcb.10308

density, i.e., an increase in cells/unit area or volume without an increase in cell proliferation [Janners and Searls, 1970; Thorogood and Hinchliffe, 1975]. Condensations are the earliest sign of the initiation of a skeletal element or elements because in some cases, more than one element can be derived from a condensation; for example, the seven bones of the lower beak of the chick embryo develop from a single condensation [Dunlop and Hall, 1995].

Mesenchymal cells of the early limb bud are surrounded by a matrix rich in hyaluronate [Toole, 1972; Toole et al., 1972] and collagen type I [Dessau et al., 1980]. It has been postulated that a pericellular hyaluronate coat prohibits interaction between mesenchymal cells, and that at the onset of condensation, an increase in hyaluronidase activity causes a reduction in hyaluronate in the extracellular matrix [Toole et al., 1989]. The reduction of extracellular matrix permits an increase in cell-cell contacts mediated by cell–cell adhesion molecules, such as N-cadherin and N-CAM, and gap junctions that facilitate intercellular communication [Coelho and Kosher, 1991a,b; Coelho et al., 1993; Cohn et al., 1995; Kelley and Fallon, 1978]. The resulting high cell density is a requirement for chondrogenesis to occur [Ahrens et al., 1977; Osdoby and Caplan, 1979] and the level of chondrogenesis is directly related to the extent of condensation [San Antonio and Tuan, 1986; Evans and Tuan, 1988].

The process of cellular condensation is directed by cell interactions with extracellular matrix proteins such as fibronectin, cell–cell adhesion mediated by membrane proteins such as N-cadherin and N-CAM, and secreted factors of the transforming growth factor-β (TGF-β) family. It has been demonstrated that TGF-β1 up-regulates expression of fibronectin, which in turn regulates N-CAM [Hall and Miyake, 1995]; in addition, N-cadherin expression may also be regulated by BMP-2, a member of the TGF-β superfamily [Haas and Tuan, 1999]. The establishment of cell–cell interactions during mesenchymal condensation is presumably involved in triggering one or more signal transduction pathways that initiate chondrogenesis. A key cell adhesion molecule implicated in this process is N-cadherin.

N-Cadherin is a member of the cadherin superfamily that consists of many members divided into six subgroups based on structural similarities. N-Cadherin, a member of the classical cadherin subgroup, is a Ca^{2+}-dependent, single transmembrane glycoprotein that mediates cell–cell adhesion by homotypic protein–protein interactions through its extracellular domain (see review by Yagi and Takeichi, 2000). The extracellular domain is composed of five tandem repeats termed cadherin repeats that form four Ca^{2+} binding sites, responsible for maintaining the extracellular domain in a stiff, rod-like conformation that forms a linear cell adhesion "zipper" with N-cadherin proteins on opposing cell membranes [Pokutta et al., 1994; Tong et al., 1994; Shapiro et al., 1995; Aberle et al., 1996]. The N-terminal repeat contains the histidine-alanine-valine (HAV) homotypic binding site common to all cadherins. The cytoplasmic domain is responsible for binding the actin cytoskeleton via the catenin family of proteins [Kemler, 1993; Jou et al., 1995; Suzuki, 1996; Alattia et al., 1999].

N-Cadherin was first identified in neural tissues, but has since been identified in many other tissues, such as somites, lens, heart, and mesenchymal tissues including developing limb mesenchyme [Hatta et al., 1987; Oberlender and Tuan, 1994b; Lee et al., 1997; Linask et al., 1998]. The importance of N-cadherin in embryonic development is underscored by the finding that N-cadherin knockout mice die by gestational day 10 primarily because of the failure of the heart to form [Radice et al., 1997]. N-Cadherin is expressed in the limb mesenchyme in a spatiotemporally specific manner suggestive of a role in cellular condensation and chondrogenesis [Oberlender and Tuan, 1994b]. Immunohistochemical localization of N-cadherin in embryonic chick limb reveals a sparsely scattered expression pattern in the central core region during the precartilage stage. Expression dramatically increases in the central core region as condensation begins and diminishes as differentiation begins. Mature cartilage is completely devoid of N-cadherin, while the cells in the surrounding perichondrium still exhibit high levels of N-cadherin [Oberlender and Tuan, 1994a]. A similar pattern of N-cadherin expression is seen in high-density micromass cultures of limb mesenchyme in vitro [Oberlender and Tuan, 1994a,b].

The spatiotemporal regulation of N-cadherin during chondrogenesis suggests that N-cadherin mediated cell–cell interactions may be important in the early stages of chondrogenesis, i.e., condensation. In fact, the NCD-2 function

blocking monoclonal antibody to chick N-cadherin [Hatta and Takeichi, 1986] inhibits cellular condensation and subsequent chondrogenesis of limb mesenchyme in vivo and in vitro [Oberlender and Tuan, 1994a,b]. This effect could be the result of inhibition of gap junction formation among the mesenchymal cells, and the interruption of crucial cell–cell interactions and intercellular communication; for example, antibodies to N-cadherin block gap junction formation in lens fiber cells [Frenzel and Johnson, 1996]. Interestingly, in high density cultures of murine C3H10T1/2 cells induced to undergo chondrogenesis with BMP-2, a concomitant increase in N-cadherin mRNA and protein was seen; also, in this chondrogenic system, treatment with an N-cadherin peptidomimic containing the HAV sequence required for homotypic interaction, inhibited chondrogenesis in a dose-dependent manner [Haas and Tuan, 1999].

In this study, we have directly examined the functional role of N-cadherin during cellular condensation and chondrogenesis in high-density chick limb micromass cultures. In these cultures, mesenchymal cells recapitulate in vitro a portion of the in vivo chondrogenic pathway, i.e., mesenchymal cells first undergo condensation forming cell aggregates that ultimately differentiate into cartilage nodules [Ahrens et al., 1977; Solursh et al., 1978]. Our approach is to transfect limb mesenchymal cells with replication competent avian retroviral-N-cadherin constructs encoding wild type and two deletion mutant constructs of N-cadherin, in order to perturb the temporal as well as qualitative characteristics of N-cadherin in these cells, and then examine the subsequent effects on chondrogenesis in micromass cultures. We report here that misexpression of wild type or mutant N-cadherin proteins inhibited chondrogenesis as assessed by alcian blue staining, [^{35}S]-sulfate incorporation, collagen type II and cartilage proteoglycan link protein immunohistochemistry. Cultures overexpressing the wild type N-cadherin protein demonstrated adequate cellular condensation, but were unable to proceed down the chondrogenic pathway, possibly due to increased cell adhesiveness resulting from the prolonged expression of N-cadherin in cells that, under normal circumstances, would be down-regulating N-cadherin expression. Both mutant N-cadherin proteins inhibited chondrogenesis at the level of cellular condensation, most likely by acting as a dominant-negative inhibitor of the endogenous N-cadherin protein. Of the two mutant proteins, the extracellular domain deletion mutant had the more severe effect on chondrogenesis by almost completely inhibiting mesenchymal cell condensation. Taken together, these data strongly support a functional role for N-cadherin in mediating mesenchymal cell chondrogenesis, specifically at the level of cellular condensation.

MATERIALS AND METHODS

Chick Embryos

Fertilized SPF-11 (C/E) White Leghorn chicken eggs were obtained from Charles River SPAFAS, Inc. (Preston, CT) and incubated at 37°C in a humidified incubator for the desired period of time.

Construction of Viral Constructs

The expand™ long template polymerase chain reaction (PCR) system (Boehringer Mannheim) was used to amplify the coding region of wild type and two deletion mutants of N-cadherin from the expression plasmids pMiwcN, pMiwcNΔ, and pMiwcN390Δ [kindly provided by Dr. Masatoshi Takeichi [Fujimori and Takeichi, 1993]] containing cDNAs for the wild type (WT), cytoplasmic deletion mutant (NΔ), and extracellular deletion mutant (390Δ) of N-cadherin, respectively, using the following primers:

Forward primer (all): 5′ ATAATA**GGCGCC**-ATGTGCCGGATAGCGGGA 3′
Reverse primer (WT & 390Δ): 5′ ATAATA-**GGCGCC**TCAGTCATCACCTCCACCGTA 3′
Reverse primer (NΔ): 5′ ATAATA**GGCGCC**T-CACAAGGATCCAGCAGTGGAGCC 3′

These primers are designed to add a Nar I restriction site (underlined and bolded) to both the 5′ and 3′ ends of the amplified cDNAs. The PCR products were digested with Nar I restriction enzyme (Promega) and purified using Clontech Chroma Spin™ columns (Palo Alto, CA) following the manufacturer's protocol. Following agarose gel electrophoresis, PCR products of 2.7, 2.6, and 1.5, corresponding to WT, NΔ, and 390Δ, respectively, were detected by ethidium bromide staining. Purified PCR products were subcloned into the replication-competent, avian leukemia virus LTR, splice

acceptor, Bryan high titer polymerase, A-envelope subgroup RCASBP(A) viral DNA [kindly provided by Dr. Steven Hughes, National Institutes of Health; Sorge and Hughes, 1982] that was linearized by digestion with Cla I restriction enzyme (Promega) and treated with calf intestinal alkaline phosphatase (CIAP; Promega) to prevent recircularization of the viral DNA. The Nar I restriction fragments were ligated to the complementary Cla I restricted vector DNA, resulting in destruction of both restriction recognition sequences. Ligated DNA was transformed into competent *Eschericia coli* JM109 cells (Promega) and grown overnight on LB agar plates containing ampicillin (50 μg/ml; Sigma). Individual bacterial colonies were picked from the agar plates and 5 ml overnight cultures grown. Plasmid DNA was isolated using the Qiagen spin mini-prep kit (Qiagen). The presence of an insert was detected by performing PCR on the plasmid DNA using the following primers that flanked the Cla I cloning site in RCASBP(A):

(forward) 5′ AACTCCATCAGCTACCACACGG 3′
(reverse) 5′ GCGTTGATGCAATTTCTATGCG-CACCCGTTCTC 3′

Plasmid DNA found to have an insert was subjected to sequencing to verify correct orientation and sequence of the insert.

Testing Viral Constructs for Production of Viral Particles and Recombinant Proteins

Chick embryo fibroblasts (CEF) were isolated as described by Morgan and Fekete [1996]. For transfection, CEFs were plated in a 12-well tissue culture dish (Costar, Corning, NY) at approximately 40% confluency, and Superfect™ (Qiagen) reagent, applied as per the manufacturer's instructions for adherent cells, was used to transfect the RCASBP(A)-N-cadherin viral constructs and empty RCASBP(A) DNA. The cells were then transferred to 75 cm^2 tissue culture flasks and expanded by splitting every 2–3 days to keep the cells proliferating, allowing for viral infection of uninfected cells. Immunohistochemistry was performed on CEFs to detect the production of viral particles using an antibody directed against the p19 gag coat protein of RCASBP(A) and recombinant protein production was examined by Western blotting of total cell lysate from CEFs (see below).

Isolation, Electroporation-Transfection, and Micromass Culture of Limb Mesenchymal Cells

Primary embryonic chick limb mesenchymal cells were isolated as described by Ahrens et al. [1977] and modified by San Antonio and Tuan [1986]. Briefly, limb buds were dissected from Hamburger-Hamilton (HH; 1951) stage 23/24 chick embryos and enzymatically dissociated into Ca^{2+}- and Mg^{2+}-free saline plus glucose (CMFSG) containing 0.1% trypsin type II (Sigma), 0.1% collagenase (Worthington Biochemical, Freehold, NJ), and 12.5% chick serum (Sigma). Isolated cells were resuspended in CMFSG plus 10% calf serum, counted using a hemacytometer in the presence of trypan blue (>95% dye exclusion), and adjusted to 40×10^6 cells/ml. DNA transfection was performed as described by [DeLise and Tuan, 2000]. Briefly, a 400 μl aliquot of the cell suspension was placed into a 0.4 cm gap width electroporation cuvette (Invitrogen). Ten microgram of the desired RCAS-N-cadherin expression constructs [R-CT, empty RCASBP(A); R-WT, wild type N-cadherin; R-NΔ, cytoplasmic deletion mutant; or R-390Δ, extracellular deletion mutant] was added to the cuvette. The β-galactosidase expression vector (pRSVLacZII; a kind gift of Dr. Lloyd Culp, Case Western Reserve University) was used for visualization and determination of transfection efficiency (see below). The cells were given a single electrical pulse using the Invitrogen Electroporator II electroporation apparatus set at 380 volts (V), 250 micro Farad (μF), and ∞ ohms (Ω). Cells were plated in 10 μl drops (unless stated otherwise) in 24-well tissue culture plates (Costar, Corning, NY), allowed to attach for 1.5 h in a humidified incubator at 37°C with 5% CO_2, and then covered with 1 ml of culture medium (CM) containing Ham's F-12, 10% fetal bovine serum (FBS; Atlanta Biological, Norcross, GA), 0.2% chick embryo extract (CEE; GibcoBRL), and 0.5% penicillin-streptomycin (Sigma). Culture medium was replaced daily.

Characterization of Electroporation-Transfection of Chick Limb Mesenchymal Cells

Transfection efficiency of electroporated cells was determined as described previously [DeLise and tuan, 2000]. Embryonic chick limb mesenchymal cells electroporated with the β-galactosidase expression plasmid (pRSVLacZII) were stained with X-Gal (Research Organics,

Cleveland, OH), and the percentage of stained cells determined microscopically.

Cell viability of electroporated cells was determined as described in [DeLise and Tuan, 2000]. Briefly, the culture medium of micromass cultures was replaced on day 1with medium containing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Molecular Probes, Eugene, OR) [Mosmann, 1983; Gerlier and Thomasset, 1986]. After incubation for 4 h, cultures were rinsed in PBS and the purple formazan crystals were dissolved with acid alcohol (0.04 N HCl in isopropanol), and A_{560} determined. The concentration of viable cells after electroporation was determined by comparison with the A_{560} values of cultures of control cells plated at known cell densities.

Immunodetection and Histochemical Procedures

Immunoblot analysis of N-cadherin. Confluent cultures of CEFs or micromass cell culture samples grown for 1–3 days, were solubilized in RIPA buffer, pH 7.4, containing 50 mM Tris, 1% NP-40, 0.1% SDS, 0.1% Na deoxycholate, 5 mM EDTA, 5 mM EGTA 150 mM NaCl, and the protease and phosphatase inhibitors phenylmethyl sulfonyl fluoride (1 mM), aprotinin (2 μg/ml), leupeptin (20 μM), sodium orthovanadate (1 mM), and sodium fluoride (40 mM) (all from Sigma) and the protein concentration determined (Micro BCA kit, Pierce Chemical, Rockford, IL). Total protein concentration in all samples was adjusted to 25 μg in a reducing sample buffer containing β-mercaptoethanol and analyzed by electrophoresis on a 7.5% polyacrylamide SDS gel. The proteins were electrophoretically transferred to 0.2 μm nitrocellulose and incubated with purified monoclonal antibody NCD-2 (see below) followed by incubation with an alkaline phosphatase conjugated goat anti-rat secondary antibody (Zymed; 1:25,000). After washing, the blots were developed histochemically with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP-NBT; Sigma).

Immunoprecipitation. Protein extracts were pre-cleared with 10% Protein A-Sepharose CL-4B (Pharmacia). Each protein (200–300 μg) was incubated in a total volume of 1.0 ml of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.2, 2% Triton X-100, 1% NP-40, 300 mM NaCl, and 3.0 mM $CaCl_2$) containing protease inhibitors [ε-amino caproic acid (50 mM), phenylmethyl sulfonyl fluoride (1 mM), benzamidine hydrochloride (5 mM)] and 0.25 μg/ml NCD-2 or β-catenin antibody for 1 h at 4°C. Immunoprecipitation was carried out by incubation with 50 μl of Protein A-Sepharose CL-4B (50% slurry); the beads were pelleted by centrifugation and then extracted by boiling in SDS–PAGE sample buffer containing β-mercaptoethanol and proteins examined by Western blotting.

Whole-mount immunohistochemistry. CEFs or micromass cultures grown for up to 3 days in culture were fixed with 4% paraformaldehyde for 20 min, rinsed, blocked with 10% normal goat serum, and incubated with various primary antibodies. Immunodetection was performed using the Zymed Histostain^{TM}-SP Kit (Zymed Laboratories, SanFrancisco, CA) followed by visualization with AEC chromogen substrate. For N-cadherin, purified NCD-2 IgG was used as the primary antibody (1:250 in TBS containing 1.5 mM Ca^{2+}). For collagen type II, cultures were first digested with hyaluronidase (300 U/ml in PBS; Sigma) for 40 min at 37°C prior to incubation with the primary monoclonal antibody, II-II6B3 (1:250 in TBS containing 0.05% Tween-20; Developmental Studies Hybridoma Bank) [Linsenmayer and Hendrix, 1980]. For cartilage proteoglycan link protein, cultures were first digested with 1.5 U/ml chondroitinase ABC (Sigma) in 50 mM Tris, pH 8.0, and 30 mM sodium acetate for 40 min at 37°C [Rapraeger et al., 1985] prior to incubation with the primary monoclonal antibody, 9/30/8-A-4 (1:250 in TBS containing 0.05% Tween-20; Developmental Studies Hybridoma Bank). For detection of the viral p19 gag protein, purified monoclonal antibody AMV-3C2 was used as the primary antibody (1:250 in TBS containing 0.05% Tween-20; Developmental Studies Hybridoma Bank).

Monoclonal antibodies. The NCD-2 rat hybridoma (a kind gift of Dr. Masatoshi Takeichi) produces a monoclonal antibody that specifically recognizes the extracellular binding region of N-cadherin and effectively inhibits normal binding function [Hatta and Takeichi, 1986]. IgG antibodies were isolated from the hybridoma supernatant by ammonium sulfate precipitation and bulk phase DEAE-Sephadex ion-exchange chromatography [Harlow and Lane, 1988], and stored at −20°C.

Peanut agglutinin (PNA) staining. To visualize cellular condensation [Zimmermann

and Thies, 1984; Aulthouse and Solursh, 1987; Milaire, 1991], micromass cultures of limb mesenchymal cells were harvested at various times during the 3-day culture period, fixed with 4% paraformaldehyde for 20 min, rinsed, and incubated with horseradish peroxidase conjugated peanut (*Arachis hypogaea*) agglutinin (Sigma; 100 μg/ml) for 30–60 min at room temperature. PNA binding was detected using the AEC colorimetric substrate (Zymed).

Alcian blue staining. Micromass cultures were grown for 3 days, rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, rinsed with PBS, and then stained overnight with 1% Alcian blue dye, pH 1.0, at room temperature [Lev and Spicer, 1964] to detect sulfated proteoglycan matrix. Cultures were then rinsed and viewed using a Wild stereomicroscope.

Metabolic Sulfate Labeling

On day 2 of culture, 1 ml of culture medium containing 2.5 μCi/ml of sodium [^{35}S]-sulfate and 1.0 μCi/ml of [^{3}H]-leucine (DuPont NEN) was added to each micromass culture plated in 24-well tissue culture plates (Corning/Costar). After 24-h, the cultures were rinsed twice with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. [^{35}S]-Sulfate and [^{3}H]-leucine incorporation in individual cultures was determined by liquid scintillation counting using a dual isotope counting program.

RESULTS

Viral Particle and Recombinant N-cadherin Protein Production in Chick Embryo Fibroblasts

A major technical requirement of this study was the expression cloning of N-cadherin cDNA sequences into the RCAS retroviral vector, which has limited size capacity for inserts [Morgan and Fekete, 1996]. The subcloning procedure undertaken here was the result of extensive optimization steps and produced stable expression constructs of relatively large cDNA, inserts (WT, 2.7 kb; NΔ, 2.6 kb; and 390Δ, 1.5 kb). PCR was used to amplify the coding region of wild type and two mutant forms of N-cadherin from expression vectors and the PCR products were subsequently sub-cloned into the replication competent avian retrovirus RCASBP(A). The wild type sequence encodes the full length N-cadherin protein (R-WT), the cytoplasmic deletion lacks the last 36 amino acids of the cytoplasmic tail that binds to β-catenin (R-NΔ), and the extracellular deletion protein is missing 390 amino acids of the extracellular domain, but contains an epitope recognized by the NCD-2 antibody (R-390Δ) (Fig. 1A).

Empty RCASBP(A), (R-CT), and RCAS-N-cadherin viral constructs were transfected into CEFs and examined for the ability of viral DNA to incorporate into the host cell and produce viral particles and recombinant N-cadherin proteins. Immunohistochemical detection of the viral p19 gag protein performed using the AMV-3C2 antibody showed positive CEF cells 1 day after transfection (Fig. 1D). The number of cells staining positive increased with culture time, indicating that viral particles were being released into the medium and infecting cells that were not transfected initially (data not shown). Non-transfected CEF cells did not stain positive with this antibody (Fig. 1E). Additionally, production of infectious viral particles was verified by treating CEFs with conditioned media from R-CT, R-WT, R-NΔ, and R-390Δ transfected CEFs and subsequent detection of the viral p19 gag coat protein (immunohistochemistry) and recombinant protein (immunoblot) in these treated cultures. All treatments resulted in positive staining for viral coat protein and produced NCD-2 immunoreactive bands on a Western blot corresponding to the size of N-cadherin (data not shown).

Western blot analysis showed that CEFs transfected with the RCAS-N-cadherin constructs produced proteins of 130, 120, and 52 kDa, corresponding to the wild type (R-WT), cytoplasmic deletion (R-NΔ), and extracellular deletion (R-390Δ) forms of N-cadherin, respectively (Fig. 1F). Uninfected CEFs have a low level of endogenous N-cadherin (Fig. 1F). In addition, the Western blot results also demonstrated that the recombinant proteins were immunoreactive to the NCD-2 antibody whose target includes the HAV homotypic binding site, and whose binding prevents N-cadherin function [Hatta and Takeichi, 1986; Oberlender and Tuan, 1994a].

Transfection Efficiency and Cell Viability of Micromass Cultures of Limb Mesenchymal Cells

Applying a recently developed method for rapid transfection of dissociated chick limb mesenchymal cells prior to plating in high density micromass culture [DeLise and Tuan, 2000], and using the β-galactosidase expression

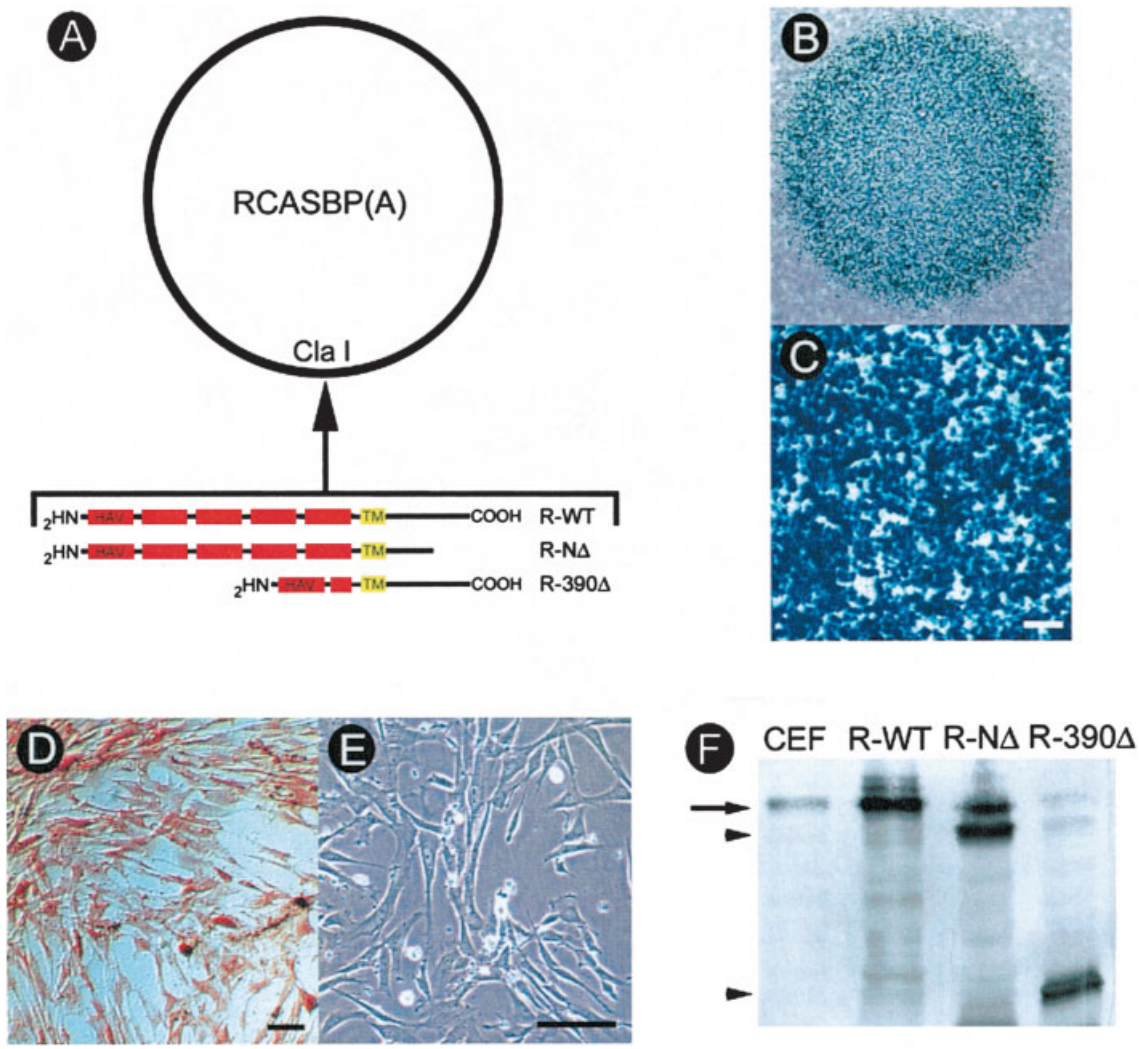

Fig. 1. Schematic diagram of RCAS-N-cadherin viral constructs and analysis of viral p19 gag and recombinant N-cadherin protein expression in chick embryo fibroblasts. **A**: Schematic diagram of the RCAS-N-cadherin viral expression constructs showing the wild type (R-WT), the cytoplasmic domain deletion mutant (R-NΔ) that lacks 36 amino acids from the C-terminal end effectively deleting the β-catenin binding site, and the extracellular domain deletion (R-390Δ) that lacks 390 amino acids from the extracellular domain but maintains the HAV homotypic binding site recognized by the NCD-2 antibody. **B**: Low magnification view of micromass culture transfected with pRSVLacZII expression plasmid and stained with X-Gal to visualize cells producing β-galactosidase (blue). The transfection efficiency averaged between 25–45%. **C**: Higher magnification view of the same X-Gal stained culture. **D–E**: High magnification view of CEF cells transfected with R-WT (D) or control cells (E) and immunostained for the expression of RCAS p19 gag coat protein. Transfected CEFs showed expression of the viral protein. **F**: Immunoblot analysis of recombinant N-cadherin protein expression in CEF cells transfected with R-WT, R-NΔ, or R-390Δ. Uninfected CEFs showed a small amount of endogenous N-cadherin expression. R-WT, R-NΔ, and R-390Δ transfected cells showed a high level of recombinant protein expression (~130, ~120, and ~52 kDa, respectively). Bars = 100 μm.

vector (pRSVLacZII), HH stage 23/24 chick limb mesenchymal cells transfected as described in Materials and Methods, showed a transfection efficiency ranging from 25–45%, as assessed by staining with X-Gal (Fig. 1B–C). More importantly, the transfected cells were able to participate in the formation of precartilage condensations and subsequent cartilage nodules [DeLise and Tuan, 2002].

Although very rapid, electroporation mediated transfection generates pores in the plasma membrane of cells, a process from which only a fraction of cells recover [Chang, 1997]. Consequently, a high mortality rate is associated with this procedure. Using the MTT cell viability assay, we determined that 40–50% of the electroporated chick mesenchyme cells are not viable as a result of the electroporation

process under our conditions. By doubling the usual cell concentration used in our micromass procedure [Ahrens et al., 1977; San Antonio and tuan, 1986; DeLise and Tuan, 2000] for the electroporated cells, the final concentration of viable cells was expected to be $20-24 \times 10^6$ cells/ml [DeLise and Tuan, 2002], approximating the known optimal concentration for chondrogenesis.

Immunoblot Analysis of Endogenous and Viral Infection Mediated Exogenous N-Cadherin Expression in Micromass Cultures of Limb Mesenchymal Cells

Immunohistochemistry for viral p19 gag protein was performed on micromass cultures electroporated with RCAS-N-cadherin viral constructs on culture days 1, 2, and 3 showed positive staining on day 1 (Fig. 2A), which increased on successive days in culture, so that by day 3, almost every cell stained positive (Fig. 2B–C). In day 3 cultures, areas devoid of staining correlated to differentiated nodules, which are rich in cartilage extracellular matrix (Fig. 2C, arrows). However, upon higher magnification, positively stained chondrocytes could be detected encased in the matrix. This observation indicated the high efficiency and completeness of the retroviral transductions.

Western blot analysis of the control micromass cultures showed a high level of N-cadherin protein on culture day 1 that decreased over subsequent days in culture, as previously described [Oberlender and Tuan, 1994b] (Fig. 2D, R-CT). Micromass cultures transfected with the RCAS-N-cadherin viral constructs showed a high level of recombinant protein expression on each culture day. Wild type (R-WT) transfected cultures showed a higher level of N-cadherin protein on culture day 1 compared to controls that increased on subsequent days in culture. The mutant transfected cultures showed immunoreactive proteins of 120 and 52 kDa, corresponding to the cytoplasmic deletion (R-NΔ) and extracellular deletion (R-390Δ), respectively. Expression level of the mutant proteins were approximately the same as that of the endogenous N-cadherin in the respective cultures on day 1, but increased considerably relative to the endogenous N-cadherin on days 2 and 3 (Fig. 2D, R-NΔ and R-390Δ). Interestingly, expression of the endogenous N-cadherin protein in R-NΔ transfected cultures did not show time-dependent down regulation as for normal limb mesenchyme micromass cultures [Oberlender and Tuan, 1994b] and seen here in the R-CT group, but instead remained higher on days 2 and 3 compared to controls. The endogenous N-cadherin protein in R-390Δ transfected cultures also did not appear to be down

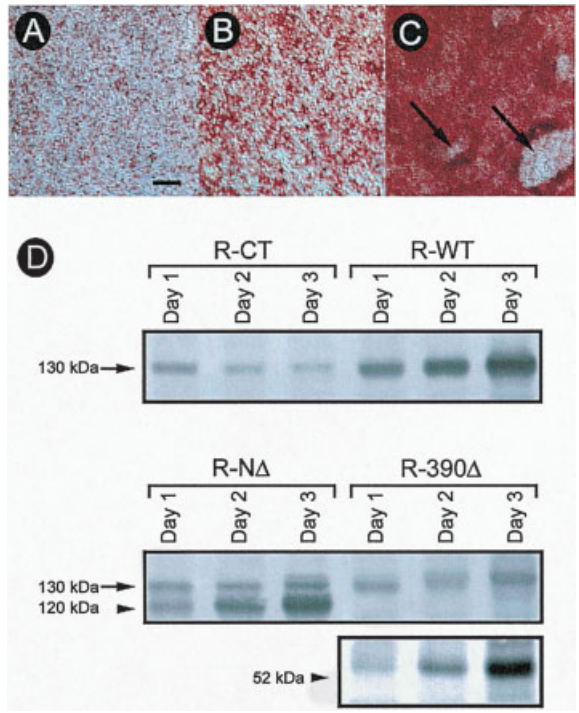

Fig. 2. Immunohistochemical detection of RCAS p19 gag coat protein and immunoblot analysis of endogenous and exogenous N-cadherin in micromass cultures as a function of culture time. **A–C**: Micromass cultures transfected with RCAS-N-cadherin viral constructs or empty vector control were stained on days 1, 2, and 3 for RCAS p19 gag coat protein expression using AMV-3C2 antibody. Presented are the results for R-390Δ; the other constructs had similar results. **A**: Day 1 culture showing high level of p19 gag expression throughout culture. **B**: Day 2 culture showing an increase in staining. **C**: Day 3 culture showing almost all cells staining positive for p19 gag protein. Differentiated, matrix-rich cartilaginous nodules were negative for staining; however, chondrocytes encased in the matrix stained positive when observed under higher magnification (arrows). Bar = 100 μm. **D**: Immunoblot analysis of N-cadherin in RCAS-N-cadherin transfected micromass cultures. In control cultures (R-CT), the level of N-cadherin protein (~130 kDa) is highest on Day 1 and then decreases on subsequent days in culture as reported previously [Oberlender and Tuan, 1994b]. R-WT, R-NΔ, and R-390Δ transfected cultures showed a high level of the recombinant proteins on Day 1 (~130, ~120, and ~52 kDa, respectively) and expression of the transgenes increased on subsequent days in culture. The endogenous N-cadherin protein in the R-NΔ and R-390Δ transfected cultures remained higher than that of the control cultures on Days 2 and 3, with a slight decrease in electrophoretic mobility in the R-390Δ transfected group on Days 2 and 3.

regulated and in addition, displayed a somewhat lower electrophoretic mobility on the gel compared to that seen in R-CT, R-N, and R-NΔ. The reason for the stabilization and lowered electrophoretic mobility of the endogenous N-cadherin in cultures expressing the mutant N-cadherin proteins is not known.

Immunoprecipitation Analysis of Endogenous and Exogenous N-Cadherin Protein

Functional cadherin depends on its interactions with β-catenin and the other catenins for the assembly of the cadherin-catenin-actin adhesion complex [Kemler, 1993]. To test the ability of the recombinant proteins to bind β-catenin, we performed immunoprecipitation analysis using a β-catenin antibody and then probed the Western blot with the NCD-2 anti-N-cadherin antibody. The R-CT and R-NΔ transfected cultures showed a strong N-cadherin band on day 1 that decreased over subsequent days and whose profile matched that of the expected N-cadherin expression pattern (Fig. 3A, R-CT and R-NΔ). The R-NΔ protein lacks the β-catenin binding domain; therefore, the pattern should mimic that of the R-CT as demonstrated. Cultures overexpressing the R-WT and R-390Δ viral constructs showed strong bands of 130 and 52 kDa on days 1 and 2, respectively, that increased further on subsequent days (Fig. 3A, R-WT and R-390Δ). The equivalence of β-catenin protein levels in the samples was assessed by immunoblot analysis of the β-catenin immunoprecipitate β-catenin antibody. The results showed similar levels of β-catenin in cultures transfected with the RCAS-N-cadherin constructs and the control cultures on each culture day (Fig. 3B).

We also performed an immunoprecipitation using the NCD-2 antibody followed by β-catenin Western blot. Again, the R-CT and R-NΔ transfected cultures showed profiles that mimicked each other because the R-NΔ protein cannot bind β-catenin (data not shown). Both the R-WT and R-390Δ transfected cultures showed increased levels of β-catenin with the highest levels occurring on day 3 (data not shown). Taken together, these data demonstrate that the recombinant proteins, i.e., WT and 390Δ, are interacting with β-catenin in a manner suggestive of a functional N-cadherin protein in terms of catenin binding as well as immunoreactivity with the NCD-2 antibody, i.e., the presence of the HAV homotypic binding site of N-cadherin.

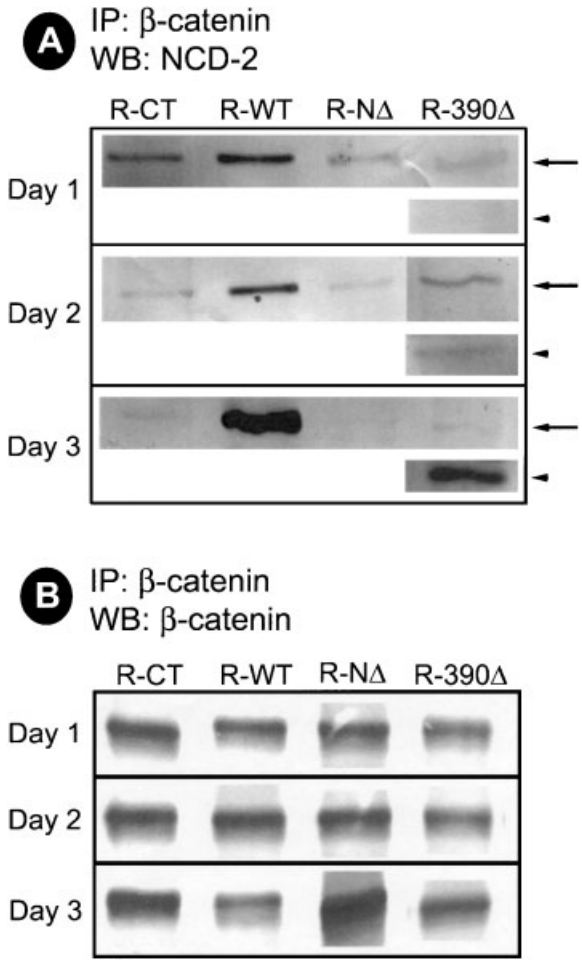

Fig. 3. Analysis of N-cadherin-β-catenin association in micromass cultures transfected with RCAS-N-cadherin constructs. Western blots (WB) of β-catenin immunoprecipitates (IP) were probed with N-cadherin or β-catenin antibodies to determine their level of association. **A**: A decrease in β-catenin association with endogenous N-cadherin from Day 1 to 3 in the R-CT, R-NΔ, and R-390Δ groups is seen and correlates well with the normal N-cadherin protein expression. A change in association is not anticipated in the R-NΔ group since the recombinant protein lacks the β-catenin binding site and is thus unable to interact with β-catenin. Cultures overexpressing the R-WT constructs show a strong N-cadherin association with β-catenin on Day 1 and this association increases dramatically on subsequent days. In R-390Δ cultures, a time-dependent increase in β-catenin association with the 52 kDa R-390Δ mutant form of N-cadherin is also seen. Arrows 130 kDa; arrowheads, 52 kDa. **B**: β-catenin Western blot analysis of the β-catenin immunoprecipitate indicates that all cultures have similar levels of β-catenin on each culture day suggesting that β-catenin level is not a limiting factor.

Whole-Mount Immunohistochemical Detection of N-Cadherin

Immunohistochemistry of micromass cultures transfected with RCAS control or RCAS-N-cadherin viral constructs was performed on days 1–3 using the NCD-2 anti-N-cadherin

antibody. Micromass cultures transfected with R-WT, R-NΔ, and R-390Δ all showed an increase in staining on culture day 1 compared to controls (Fig. 4A–D). A continued increase in staining intensity was observed in these same groups on subsequent days in culture compared to controls (data not shown), supporting the Western blot data.

Effect of Expressing Wild Type and Mutant Forms of N-Cadherin on Cellular Condensation

The effect of overexpressing N-cadherin on cellular condensation was examined histochemically by staining with PNA, a galactose-specific lectin that recognizes β-D-Gal-NAc-D-Gal, a cell membrane carbohydrate moiety found on condensing chondroprogenitor cells [Zimmermann and Thies, 1984; Aulthouse and Solursh, 1987; Davies et al., 1990; Milaire, 1991; Oakley et al., 1994]. On day 1, R-CT, R-WT, and R-NΔ all showed uniform PNA staining (Fig. 5A–C), while the R-390Δ cultures showed a slight decrease in PNA staining compared to all other cultures (compare Fig. 5D to A–C). By day 2, R-CT and R-WT showed similar PNA staining (Fig. 5E–F) while R-NΔ and R-390Δ showed a decrease in PNA staining compared to the controls (compare Fig. 5G–H to E), indicating an inhibition of cellular condensation. On culture day 3, R-WT has a slight decrease in PNA staining compared to R-CT (compare Fig. 5J to I). R-NΔ has significantly less PNA staining compared to controls (compare Fig. 5K to I) and R-390Δ has essentially no PNA staining (Fig. 5L), indicating the absence of any cellular condensation.

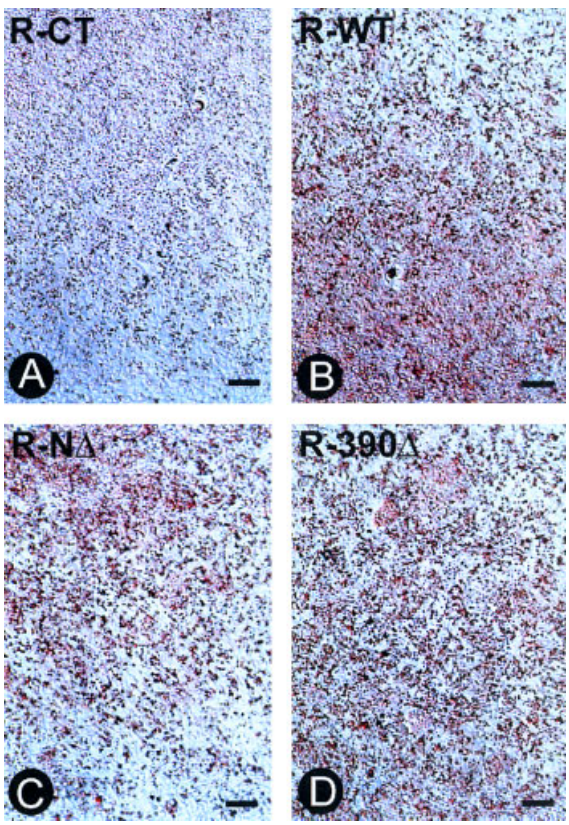

Fig. 4. Immunohistochemical detection of N-cadherin protein in micromass cultures transfected with RCAS-N-cadherin constructs. All cultures were stained on Day 1 for N-cadherin protein using the monoclonal antibody NCD-2. **A**: Control cultures showed staining for N-cadherin protein throughout the culture. **B–D**: Micromass cultures transfected with R-WT, R-NΔ, and R-390Δ constructs showed an increase in N-cadherin staining compared to control cultures (A; R-CT). Bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of Overexpressing Wild Type and Mutant Forms of N-Cadherin on Chondrogenesis

Alcian blue staining. Micromass cultures overexpressing the RCAS-N-cadherin viral constructs showed an inhibition of cartilage differentiation on day 3 as assayed by Alcian blue staining. Cultures expressing R-WT showed decreased alcian blue staining compared to the control cultures (compare Fig. 6B to A). Expression of both mutants resulted in significant decrease in Alcian blue staining compared to the controls, with R-390Δ having the greatest inhibitory effect by almost completely eliminating cartilage formation (compare Fig. 6C–D to A).

[^{35}S]-sulfate incorporation. A statistically significant decrease in metabolic [^{35}S]-sulfate incorporation relative to total protein synthesis ([^{3}H]-leucine incorporation) was seen in R-WT, R-NΔ, and R-390Δ overexpressing cultures compared to the controls (R-CT) (Fig. 6E).

Immunohistochemistry of cartilage matrix. Chondrogenesis was assessed by immunostaining for collagen type II, the major collagenous protein in cartilage matrix [Upholt and Olsen, 1991], and cartilage proteoglycan link protein [Stirpe and Goetinck, 1989]. Day 3 micromass cultures overexpressing the wild type or either deletion mutant N-cadherin protein showed decreased staining for both collagen type II and link protein compared to controls (compare Fig. 7B–D to A and Fig. 7F–H to E, respectively). Cultures overexpressing R-390Δ had the most severe inhibition of

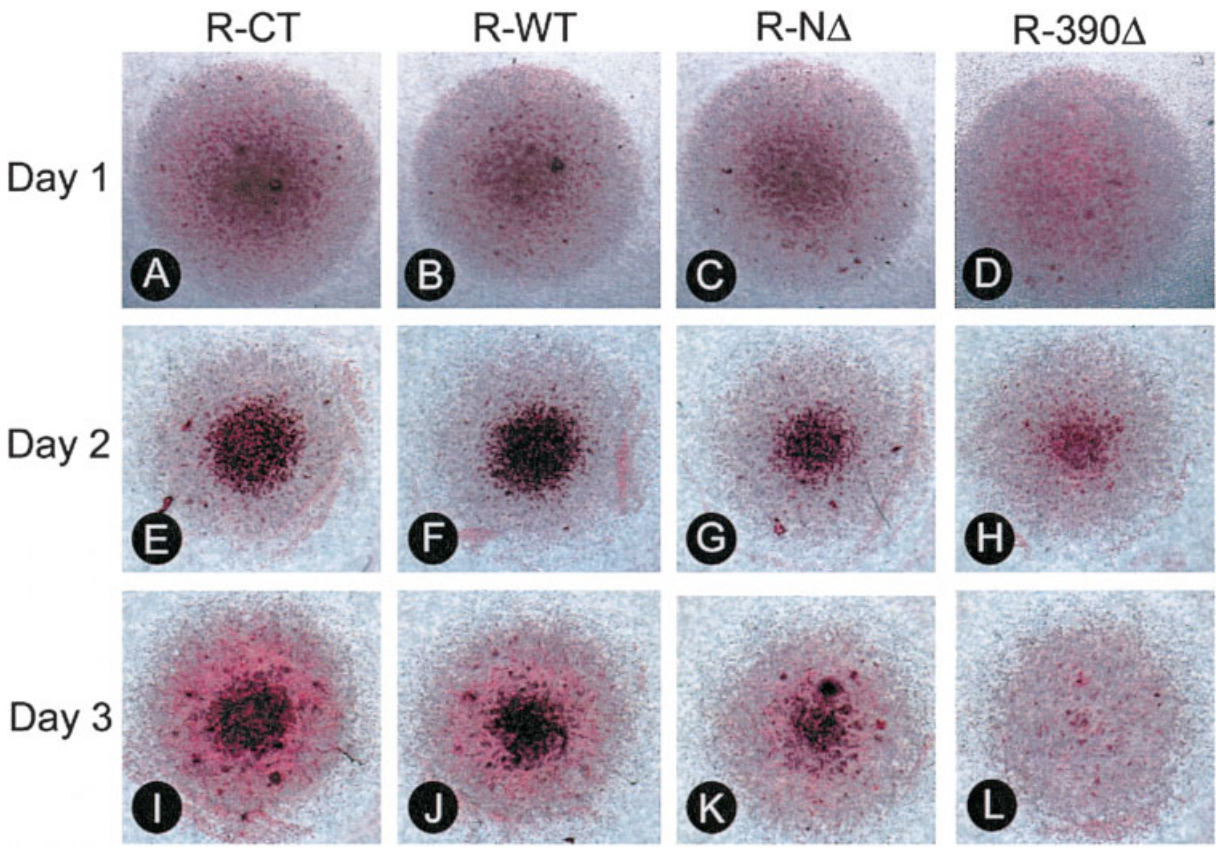

Fig. 5. Analysis of cellular condensation in chick limb micromass cultures transfected with RCAS-N-cadherin constructs demonstrated by PNA staining. **A–D**: On Day 1, PNA staining was uniform in R-CT, R-WT, and R-NΔ groups while a slight decrease in PNA staining was seen in the R-390Δ group. **E–H**: By Day 2, a decrease in PNA staining is seen in the R-NΔ and R-390Δ groups G, H: compared to controls (E). Cultures overexpressing the wild type N-cadherin (R-WT) (F) showed PNA staining similar to that of the control (E). **I–L**: On Day 3, the control cultures (I) and cultures overexpressing wild type N-cadherin (J; R-WT) displayed similar PNA staining again while R-NΔ cultures (K) showed a decrease in PNA staining compared to the controls (I). R-390Δ cultures (J) showed almost no PNA staining on Day 3.

staining for both matrix proteins with staining barely above background. These data further support and confirm the Alcian blue histochemical and [^{35}S]-sulfate incorporation data.

DISCUSSION

In this study, we have used a replication competent avian retrovirus to efficiently express recombinant wild type and deletion mutant N-cadherin proteins in embryonic chick limb bud micromass cultures. The results clearly show that overexpressing wild type or deletion mutant forms of N-cadherin that perturb extracellular homotypic binding or binding to the catenin proteins and therefore to the actin cytoskeleton, inhibits chondrogenesis of limb mesenchymal cells in vitro. Overexpressing of wild type N-cadherin did not appear to affect cellular condensation on the basis of PNA staining, whereas overexpression of either mutant N-cadherin protein suppressed cellular condensation beginning on day 1 for R-390Δ and day 2 for R-NΔ. Although cellular condensation appeared normal, the wild type N-cadherin expressing cultures showed a reduction in differentiated cartilage, on the basis of alcian blue staining, collagen type II and link protein production, and [^{35}S]-sulfate incorporation into newly synthesized extracellular matrix. Cultures expressing either N-cadherin mutant also showed a significant reduction in differentiated cartilage compared to controls using the same markers for cartilage differentiation; however, the degree of inhibition was greater for the extracellular deletion mutant (R-390Δ) compared to the cytoplasmic deletion mutant (R-NΔ). Given the developmentally regulated expression pattern of N-cadherin mRNA and protein in the embryonic limb, with maximal expression occurring at cellular condensation [Oberlender

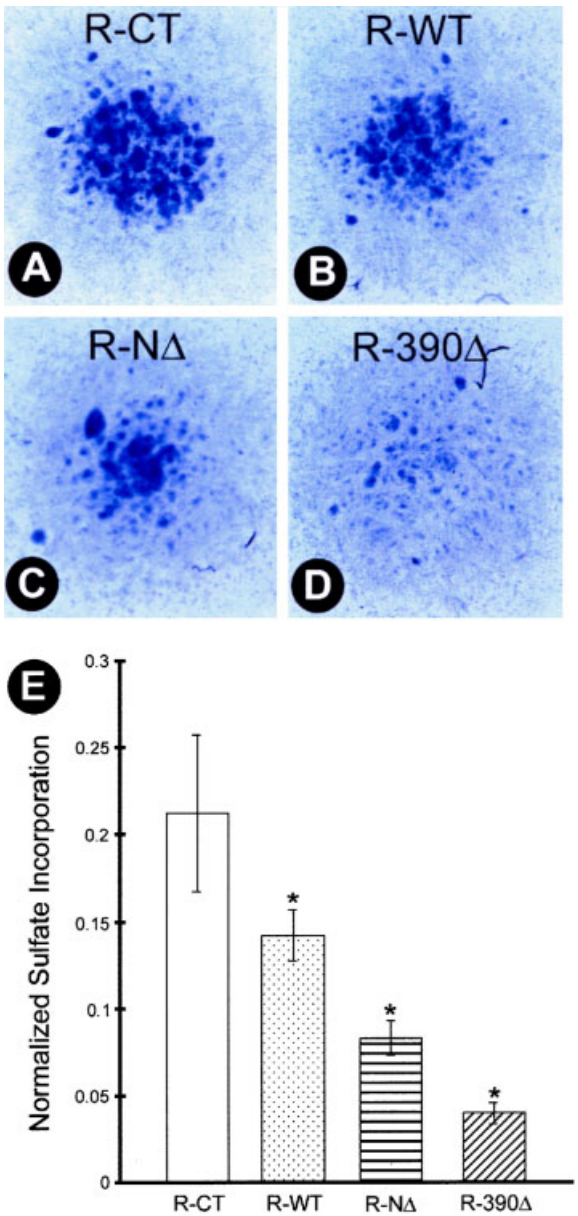

Fig. 6. Analysis of chondrogenesis in chick limb micromass cultures transfected with RCAS-N-cadherin constructs demonstrated by Alcian blue staining and metabolic sulfate incorporation. All cultures were stained on Day 3. Cultures overexpressing wild type N-cadherin **(B**; R-WT**)** and both mutant proteins **(C**; R-NΔ and **D**; R-390Δ**)** showed decreased Alcian blue staining compared to controls **(A**; R-CT**)**. A statistically significant decrease in sulfate incorporation on Day 3 was seen in all treated cultures (R-WT, R-NΔ, and R-390Δ) compared to controls **(E)**. n = 15, $^*P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and Tuan, 1994b], the in vitro effects observed here are thus consistent for a functional role for this cell–cell adhesion molecule in chondrogenesis, specifically during the cellular condensation phase.

The RCAS N-cadherin constructs described here were robust and resulted in high efficiency transfection and stable expression of the N-cadherin constructs in HH stage 23/24 chick embryos. Thus, mesenchymal cells show a high level of virus expression, on the basis of p19 gag protein, on culture day 1 and an increase in expression on each successive day in culture. This correlates well with the expression of the transgenes; R-WT overexpressing cells show an elevated level of expression of wild type protein (~130 kDa) on day 1 compared to controls and the expression continues to increase on each successive day. The same expression pattern is seen for the recombinant proteins in R-NΔ (~120 kDa) and R-390Δ (~52kDa) transfected cultures (Fig. 2D). The temporal expression profile seen in these cultures is directly opposite to that of endogenous N-cadherin gene in the controls, and to that observed by [Oberlender and Tuan, 1994b], i.e., a high level of N-cadherin protein on day 1 and a decrease in expression on subsequent days. Interestingly, endogenous N-cadherin protein in the R-NΔ and R-390Δ groups appears to be stabilized since the expression on days 2 and 3 is higher than that of the controls on these days. Also, endogenous N-cadherin in the R-390Δ group has changed its electrophoretic mobility on days 2 and 3 as seen by the reduced migration of this protein on the Western blot. The reason for the stabilization and reduced electrophoretic mobility of the endogenous N-cadherin protein in the cultures expressing the mutant N-cadherin proteins is not known, although one possible alteration is the level of glycosylation.

Our working hypothesis is that the overexpression of wild type N-cadherin protein will enhance chondrogenesis by increasing the size and/or number of cellular condensations, and subsequently, the amount of differentiated nodules. On the other hand, the mutants would act as "dominant-negative inhibitors" of the endogenous wild type N-cadherin protein. One possibility is that the R-390Δ mutant (i.e., extracellular deletion) would dimerize with the endogenous wild type N-cadherin and prevent homotypic interactions or it could dimerize with another mutant and compete with the endogenous N-cadherin proteins for β-catenin binding and subsequent linkage to the cytoskeleton. The R-NΔ mutant (i.e., cytoplasmic deletion) would also either homodimerize or heterodimerize with the wild type N-cadherin protein.

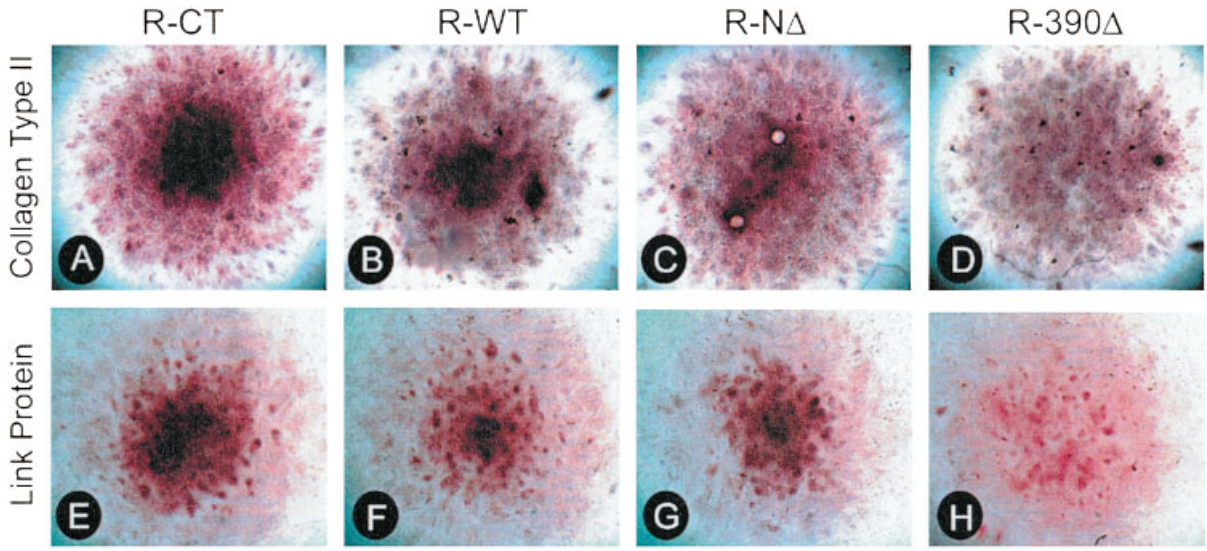

Fig. 7. Immunohistochemical detection of collagen type II and cartilage proteoglycan link protein in chick limb micromass cultures transfected with RCAS-N-cadherin constructs. All cultures were stained on Day 3 for collagen type II using the monoclonal antibody II-II6B3 and link protein using the monoclonal antibody 9/30/8-A-4. A decrease in both collagen type II and link protein staining was seen in cultures overexpressing wild type N-cadherin (**B**, **F**; R-WT), and both mutant expressing cultures (**C**, **G**; R-NΔ and **D**, **H**; R-390Δ) compared to controls (**A**, **E**; R-CT).

Homodimers could compete with that of wild type dimers for interaction with dimers on opposing cells; upon binding, signal transduction would be inhibited due to the lack of cytosolic interaction with the catenin proteins and subsequent linkage to the actin cytoskeleton. Although heterodimers have one functional N-cadherin, they would most likely function similarly because they would be unable to form the same adhesion complex as wild type proteins. The effect of overexpressing recombinant N-cadherin proteins on cellular condensation of limb mesenchyme in vitro, PNA staining was used to detect the condensing chondroprogenitor cells [Zimmermann and Thies, 1984; Aulthouse and Solursh, 1987; Davies et al., 1990; Milaire, 1991; Oakley et al., 1994].

Our data show that stable overexpression of wild type N-cadherin in limb mesenchymal cells in vitro did not affect cellular condensation but inhibited chondrogenic differentiation assayed by histological Alcian blue staining, metabolic [^{35}S]-sulfate incorporation, and immunohistological detection of cartilage proteins collagen type II and cartilage proteoglycan link protein. The amount of PNA staining in these cultures was similar to control cultures on each culture day with only a slight decrease on day 3. Because of the high expression level of N-cadherin protein during cellular condensation [Oberlender and Tuan, 1994b], one might expect a direct relationship between the amount of N-cadherin in a culture and the amount of cellular condensation. Indeed, we have previously observed that transient overexpression of wild type N-cadherin in limb mesenchyme in vitro, in a manner similar to the temporal profile of endogenous N-cadherin gene expression, increased the amount of cellular condensation and also reduced the cell density requirement for initiation of condensation [DeLise and Tuan, 2002]. However, in the present study, we are able to maintain high levels of N-cadherin protein in the mesenchymal cell cultures throughout the entire culture period using a replication competent retrovirus, i.e., recombinant protein expression was higher on day 1 compared to controls and the expression levels significantly increased on subsequent days in culture while that of the controls decreased.

It is interesting that Haas and Tuan [1999] have recently shown that stably transfected C3H10T1/2 cells expressing N-cadherin at different levels compared to controls had differential effects on chondrogenesis when induced by BMP-2. Cells with a twofold increase in N-cadherin demonstrated increased chondrogenesis, while a fourfold increase in N-cadherin resulted in an inhibition of chondrogenesis

compared to controls. Additionally, inhibition of chondrogenesis in chick limb bud micromass cultures as a result of retrovirally transduced overexpression of Wnt-7a was also accompanied by elevated N-cadherin expression throughout the entire culture period [Rudnicki and Brown, 1997; Stott et al., 1999; Tufan and Tuan, 2001] Blockage of chondrogenesis by inhibiting protein kinase A (PKA) [Yoon et al., 2000] and p38 [Oh et al., 2000] was accompanied by sustained N-cadherin expression into the normally chondrogenic phase of limb mesenchyme micromass cultures. In another study, Fujimori et al. [1990], showed perturbed histogenesis of tissues in *Xenopus* embryos after injection of N-cadherin mRNA into one blastomere of a 2-cell-stage *Xenopus* embryo. Taken together, these data suggest that quantitative differences in N-cadherin expression can control morphogenesis of different tissues.

The retroviral expression of wild type N-cadherin caused an increase in N-cadherin protein on day 1 compared to controls with an even larger increase on Days 2 and 3. This prevented the down-regulation of N-cadherin protein normally seen in control cultures. Therefore, the tight, developmentally regulated expression of N-cadherin during chondrogenesis [Oberlender and Tuan, 1994b] was perturbed. The effects seen may be attributed to mesenchymal cells forming tighter connections [Nose et al., 1988; Miyatani et al., 1989] and thus being unable to continue down the chondrogenic pathway. Mesenchymal cells must undergo a decrease in cell–cell adhesion following cell condensation that is accompanied by a cell shape change from fibroblastic to a more rounded morphology. It is thus crucial that N-cadherin be down regulated to permit normal differentiation progression. In fact, the expression of N-cadherin is very dynamic during embryonic development and these changes are always correlated with various cell behaviors [see review by Takeichi, 1988].

The cadherin family of proteins mediate cell adhesion through their extracellular domain that is in part regulated by the interaction of the cytoplasmic domain with the actin cytoskeleton [Nagafuchi and Takeichi, 1988; Fujimori et al., 1990; Fujimori and Takeichi, 1993]. Expression of mutant cadherin proteins in various systems has confirmed the importance of cadherins in cell adhesion and normal tissue morphogenesis [Hatta and Takeichi, 1986; Kintner, 1992; Dufour et al., 1994]. In addition, use of these deletion mutant constructs in C3H10T1/2 cells, epithelial cells, and L-cells showed inhibition of N-cadherin mediated cell adhesion or aggregation [Fujimori et al., 1990; Fujimori and Takeichi, 1993; Haas and Tuan, 1999]. Our results show that overexpression of both mutant N-cadherin proteins had an inhibitory effect on limb mesenchyme cellular condensation as demonstrated by an inhibition in PNA staining beginning on day 1 for R-390Δ and day 2 for R-NΔ transfected cultures. Consequently, chondrogenic differentiation was also inhibited.

The N-cadherin mutant proteins may be exerting their effect by either displacing endogenous N-cadherin in the adhesion complex, competing for cytosolic proteins, or by down-regulating the expression of the endogenous N-cadherin protein. Our immunoprecipitation data showed that with the increase in expression of the R-390Δ mutant is accompanied by an increase in β-catenin association with this mutant protein (note: R-NΔ lacks the β-catenin binding site). Thus, the cytosolic proteins are unlikely to be the limiting components in this interaction. Also, Fujimori and Takeichi [1993] showed that the cytoplasmic proteins were present in excess in epithelial cells after transfection with the N-cadherin extracellular mutant. We also did not see a decrease in endogenous N-cadherin protein after transfection with either N-cadherin mutant as described by [Nieman et al., 1999] as the mechanism contributing to reduced cell adhesion. Therefore, it appears that the N-cadherin mutants are functional in replacing the endogenous N-cadherin in the adhesion complex.

N-Cadherin proteins dimerize in the plasma membrane and initiate interactions with dimers on opposing cells. It has been reported that the cytoplasmic tail of N-cadherin has sufficient information to initiate lateral clustering or dimerization without the need for the extracellular domain [Katz et al., 1998]. Therefore, the extracellular deletion mutant (R-390Δ) with an intact cytoplasmic domain can dimerize with either mutant or wild type N-cadherin protein. Since we are able to overexpress the mutant protein at levels well above that of the endogenous N-cadherin protein, three possible scenarios could occur. First, the mutant proteins will only dimerize with themselves as will the wild type N-cadherin proteins (homodimers); second, the mutant and wild type N-cadherin

proteins will dimerize with each other (heterodimers); or third, a mixture of homo- and heterodimers will form. Since N-cadherin only requires the cytoplasmic tail to dimerize, most likely both homo- and heterodimers will form with a preponderance of mutant homodimers since this protein is present in such excess. These cells will thus be significantly compromised in N-cadherin mediated cell-cell adhesion, resulting in inhibition of cellular condensation and subsequent chondrogenesis.

Another possibility is that the overexpression of the mutant protein is acting as a "sink" for free β-catenin proteins, and therefore, preventing β-catenin proteins from interacting with other cadherin proteins that may play a role during chondrogenesis. For example, cadherin-11 is expressed in all mesenchymal cells regardless of their origin [Hoffmann and Balling, 1995; Kimura et al., 1995; Simonneau et al., 1995], and studies have shown that N-cadherin and cadherin-11 expression are complementary in some tissues, including condensing mesenchyme of the limb and somites [Kimura et al., 1995]. Although cadherin-11 has not been shown to play a direct role in chondrogenesis, a recent study investigating somite formation showed that N-cadherin null mice had fragmented somites whereas cadherin-11 null mice showed no structural anomalies. However, mice homozygous for both N-cadherin and cadherin-11 mutations had more fragmented somites than N-cadherin deficient mice alone [Horikawa et al., 1999], suggesting that the two cadherins may both be involved in the formation of somites with cooperative but different functions. It has been suggested that cadherin-11 may play a role in mesenchymal cell sorting [Kimura et al., 1995].

The cytoplasmic domain of cadherins interacts with many cytosolic proteins that have been implicated in regulating the adhesion complex either by phosphorylating or dephosphorylating key members of the adhesion complex that in turn regulate assembly or disassembly of the complex. Some of the proteins implicated are PTP1B [Balsamo et al., 1998], $p120^{catenin}$ [Ohkubo and Ozawa, 1999], and Rho family of GTPases [Kaibuchi et al., 1999]. It appears that the degree to which the cytoplasmic domain is deleted will determine the effect it will have. Deleting the β-catenin binding site in E-cadherin has been reported to prevent epithelial cells from aggregating [Nagafuchi et al., 1987], but deletion of the cytoplasmic domain to seven amino acids or fewer (deletes $p120^{catenin}$ binding site) restores the ability of cells to aggregate in an E-cadherin dependent manner [Ozawa and Kemler, 1998]. Therefore, it appears that different regions of the cytoplasmic domain have different effects on cell adhesion. The mutant used in this study has the β-catenin binding site deleted but maintains the $p120^{catenin}$ binding site. Consequently, any regulation of the adhesion complex performed by $p120^{catenin}$ can still occur, whereas signal transduction dependent on linkage to the actin cytoskeleton via β-catenin will be inhibited.

Both genetic mutations and teratogens can affect the cellular condensation process that ultimately results in skeletal anomalies. In this study, we have provided evidence directly linking N-cadherin to cellular condensation and for the required temporal regulation of N-cadherin during chondrogenesis. Specifically, by overexpressing the wild type N-cadherin protein in limb mesenchymal cells in vitro, we have demonstrated the requirement for the down-regulation of N-cadherin after condensation to permit the procession of cells into the differentiation process. In addition, exogenous expression of mutant N-cadherin proteins lacking either the homotypic binding domain or the β-catenin binding site demonstrates a requirement of both homotypic binding and the formation of the intracellular adhesion complex for N-cadherin activity in mesenchymal cell condensation and chondrogenesis. We are currently examining the exact signaling events responsible for N-cadherin regulation.

ACKNOWLEDGMENTS

The authors thank Dr. M. Takeichi for supplying the N-cadherin cDNAs (pMiwcN, pMiwcNΔ, and pMiwcN390Δ) and the NCD-2 hybridoma cell line; Dr. S. Hughes for supplying the RCASBP(A) DNA; Jessica Moss for her technical assistance; and Lauren Reed for manuscript preparation. AMD was a trainee supported by NIEHS training grant T32ES07282.

REFERENCES

Aberle H, Schwartz H, Kemler R. 1996. Cadherin–catenin complex: Protein interactions and their implications for cadherin function. J Cell Biochem 61:514–523.

Ahrens PB, Solursh M, Reiter RS. 1977. Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.

Alattia J, Kurokawa H, Ikura M. 1999. Structural view of cadherin-mediated cell–cell adhesion. Cell Mol Life Sci 55:359–367.

Aulthouse AL, Solursh M. 1987. The detection of a pre-cartilage, blastema-specific marker. Dev Biol 120:377–384.

Balsamo J, Arregui C, Leung T, Lilien J. 1998. The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin-actin linkage. J Cell Biol 143:523–532.

Chang DC. 1997. Experimental strategies in efficient transfection of mammalian cells. Electroporation Meth Mol Biol 62:307–318.

Coelho CN, Kosher RA. 1991a. Gap junctional communication during limb cartilage differentiation. Dev Biol 144: 47–53.

Coelho CN, Kosher RA. 1991b. A gradient of gap junctional communication along the anterior-posterior axis of the developing chick limb bud. Dev Biol 148:529–535.

Coelho CN, Sumoy L, Rogina B, Upholt WB, Kosher RA. 1993. Roles of the chicken homeobox-containing genes *GHox-8*, *GHox-7*, and *GHox-4.6* in pattern formation during limb development. Prog Clin Biol Res 383A: 61–70.

Cohn MJ, Izpisua-Belmonte JC, Abud H, Heath JK, Tickle C. 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. Cell 80:739–746.

Davies JA, Cook GM, Stern CD, Keynes RJ. 1990. Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. Neuron 4:11–20.

DeLise AM, Tuan RS. 2000. Electroporation-mediated DNA transfection of embryonic chick limb mesenchymal cells. In: Tuan RS, Lo CW, editors. Developmental biology protocols. Vol. 3: Humana Press, Inc. pp 377–382.

DeLise AM, Tuan RS. Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro. Dev Dyn, in press.

Dessau W, von der Mark H, von der Mark K, Fischer S. 1980. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. J Embryol Exp Morphol 57:51–60.

Dufour S, Saint-Jeannet JP, Broders F, Wedlich D, Thiery JP. 1994. Differential perturbations in the morphogenesis of anterior structures induced by overexpression of truncated XB- and N-cadherins in Xenopus embryos. J Cell Biol 127:521–535.

Dunlop LL, Hall BK. 1995. Relationships between cellular condensation, preosteoblast formation, and epithelial-mesenchymal interactions in initiation of osteogenesis. Int J Dev Biol 39:357–371.

Evans MS, Tuan RS. 1988. Cellular condensation and collagen Type II expression during chondrogenesis in vitro. J Cell Biol 107:163a.

Fell HB. 1925. The histogenesis of cartilage and bone in the long bones of the embryonic fowl. J Morphol 40:417–451.

Fell HB, Canti RG. 1935. Experiments on the development in vitro of the avian knee joint. Proc R Soc Lond B Biol Sci 166:316–351.

Frenzel EM, Johnson RG. 1996. Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. Dev Biol 179:1–16.

Fujimori T, Takeichi M. 1993. Disruption of epithelial cell–cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. Mol Biol Cell 4:37–47.

Fujimori T, Miyatani S, Takeichi M. 1990. Ectopic expression of N-cadherin perturbs histogenesis in Xenopus embryos. Development 110:97–104.

Gerlier D, Thomasset N. 1986. Use of MTT colorimetric assay to measure cell activation. J Immunol Meth 94:57–63.

Gruneberg H. 1963. The pathology of development: A Study of Inherited Skeletal Disorders in Animals. Oxford: Blackwell Scientific Publications.

Haas AR, Tuan RS. 1999. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: II. Stimulation by bone morphogenetic protein-2 requires modulation of N-cadherin expression and function. Differentiation 64:77–89.

Hall BK, Miyake T. 1992. The membranous skeleton: The role of cell condensations in vertebrate skeletogenesis. Anat Embryol 186:107–124.

Hall BK, Miyake T. 1995. Divide, accumulate, differtiate: Cell condensation in skeletal development revisited. Int J Dev Biol 39:881–893.

Hall BK, Miyake T. 2000. All for one and one for all: Condensations and the initiation of skeletal development. Bioessays 22:138–147.

Harlow E, Lane D. 1988. Antibodies: A laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hatta K, Takeichi M. 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature 320:447–449.

Hatta K, Takagi S, Fujisawa H, Takeichi M. 1987. Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev Biol 120:215–227.

Hoffmann I, Balling R. 1995. Cloning and expression analysis of a novel mesodermally expressed cadherin. Dev Biol 169:337–346.

Horikawa K, Radice G, Takeichi M, Chisaka O. 1999. Adhesive subdivisions intrinsic to the epithelial somites. Dev Biol 215:182–189.

Janners MY, Searls RL. 1970. Changes in rate of cellular proliferation during the differentiation of cartilage and muscle in the mesenchyme of the embryonic chick wing. Dev Biol 23:136–165.

Jou TS, Stewart DB, Stappert J, Nelson WJ, Marrs JA. 1995. Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. Proc Natl Acad Sci USA 92:5067–5071.

Kaibuchi K, Kuroda S, Fukata M, Nakagawa M. 1999. Regulation of cadherin-mediated cell–cell adhesion by the Rho family GTPases. Curr Opin Cell Biol 11:591–596.

Katz BZ, Levenberg S, Yamada KM, Geiger B. 1998. Modulation of cell–cell adherens junctions by surface clustering of the N-cadherin cytoplasmic tail. Exp Cell Res 243:415–424.

Kelley RO, Fallon JF. 1978. Identification and distribution of gap junctions in the mesoderm of the developing chick limb bud. J Embryol Exp Morphol 46:99–110.

Kemler R. 1993. From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet 9:317–321.

Kimura Y, Matsunami H, Inoue T, Shimamura K, Uchida N, Ueno T, Miyazaki T, Takeichi M. 1995. Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos. Dev Biol 169:347–358.

Kintner C. 1992. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell 69:225–236.

Lee MM, Fink BD, Grunwald GB. 1997. Evidence that tyrosine phosphorylation regulates N-cadherin turnover during retinal development. Dev Genet 20:224–234.

Lev R, Spicer SS. 1964. Specific staining of sulfate groups with Alcian blue at low pH. J Histochem Cytochem 12:309.

Linask KK, Ludwig C, Han MD, Liu X, Radice GL, Knudsen KA. 1998. N-cadherin/catenin-mediated morphoregulation of somite formation. Dev Biol 202:85–102.

Linsenmayer TF, Hendrix MJ. 1980. Monoclonal antibodies to connective tissue macromolecules: Type II collagen. Biochem Biophys Res Commun 92:440–446.

Milaire J. 1991. Lectin binding sites in developing mouse limb buds. Anat Embryol 184:479–488.

Miyatani S, Shimamura K, Hatta M, Nagafuchi A, Nose A, Matsunaga M, Hatta K, Takeichi M. 1989. Neural cadherin: Role in selective cell–cell adhesion. Science 245:631–635.

Morgan BA, Fekete DM. 1996. Manipulating gene expression with replication-competent retroviruses. In: Bronner-Fraser M, editor. Methods in cell biology: Methods in avian embryology. San Diego: Academic Press;. pp 185–218.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Meth 65:55–63.

Mundlos S, Olsen BR. 1997a. Heritable diseases of the skeleton. Part I: Molecular insights into skeletal development-transcription factors and signaling pathways. FASEB J 11:125–132.

Mundlos S, Olsen BR. 1997b. Heritable diseases of the skeleton. Part II: Molecular insights into skeletal development-matrix components and their homeostasis. FASEB J 11:227–233.

Nagafuchi A, Takeichi M. 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J 7:3679–3684.

Nagafuchi A, Shirayoshi Y, Okazaki K, Yasuda K, Takeichi M. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. Nature 329: 341–343.

Nieman MT, Kim JB, Johnson KR, Wheelock MJ. 1999. Mechanism of extracellular domain-deleted dominant negative cadherins. J Cell Sci 112:1621–1632.

Nose A, Nagafuchi A, Takeichi M. 1988. Expressed recombinant cadherins mediate cell sorting in model systems. Cell 54:993–1001.

Oakley RA, Lasky CJ, Erickson CA, Tosney KW. 1994. Glycoconjugates mark a transient barrier to neural crest migration in the chicken embryo. Development 120:103–114.

Oberlender SA, Tuan RS. 1994a. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development 120:177–187.

Oberlender SA, Tuan RS. 1994b. Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. Cell Adhes Commun 2:521–537.

Oh C, Chang S, Yoon Y, Lee S, Lee Y, Kang S, Chun J. 2000. Opposing role of mitogen-activated protein kinase subtypes, erk-1/2, and p38, in the regulation of chondrogenesis of mesenchymes. J Biol Chem 275:5613–5619.

Ohkubo T, Ozawa M. 1999. p120(ctn) binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. J Biol Chem 274:21409–21415.

Osdoby P, Caplan AI. 1979. Osteogenesis in cultures of limb mesenchymal cells. Dev Biol 73:84–102.

Ozawa M, Kemler R. 1998. The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. J Cell Biol 142:1605–1613.

Pokutta S, Herrenknecht K, Kemler R, Engel J. 1994. Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. Eur J Biochem 223:1019–1026.

Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO. 1997. Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64–78.

Rapraeger A, Jalkanen M, Endo E, Koda J, Bernfield M. 1985. The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. J Biol Chem 260:11046–11052.

Rudnicki JA, Brown AM. 1997. Inhibition of chondrogenesis by *Wnt* gene expression in vivo and in vitro. Dev Biol 185:104–118.

San Antonio JD, Tuan RS. 1986. Chondrogenesis of limb bud mesenchyme in vitro: Stimulation by cations. Dev Biol 115:313–324.

Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand JF, Als-Nielsen J, Colman DR, Hendrickson WA. 1995. Structural basis of cell–cell adhesion by cadherins [see comments]. Nature 374: 327–337.

Simonneau L, Kitagawa M, Suzuki S, Thiery JP. 1995. Cadherin 11 expression marks the mesenchymal phenotype: Towards new functions for cadherins? Cell Adhes Commun 3:115–130.

Solursh M, Ahrens PB, Reiter RS. 1978. A tissue culture analysis of the steps in limb chondrogenesis. In Vitro 14: 51–61.

Sorge J, Hughes SH. 1982. Splicing of intervening sequences introduced into an infectious retroviral vector. J Mol Appl Genet 1:547–559.

Stirpe NS, Goetinck PF. 1989. Gene regulation during cartilage differentiation: Temporal and spatial expression of link protein and cartilage matrix protein in the developing limb. Development 107:23–33.

Stott NS, Jiang TX, Chuong CM. 1999. Successive formative stages of precartilaginous mesenchymal condensations in vitro: Modulation of cell adhesion by Wnt-7A and BMP-2. J Cell Phys 180:314–324.

Suzuki ST. 1996. Structural and functional diversity of cadherin superfamily: Are new members of cadherin superfamily involved in signal transduction pathway? J Cell Biochem 61:531–542.

Takeichi M. 1988. The cadherins: Cell–cell adhesion molecules controlling animal morphogenesis. Development 102:639–655.

Thorogood PV, Hinchliffe JR. 1975. An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. J Embryol Exp Morphol 33:581–606.

Tong KI, Yau P, Overduin M, Bagby S, Porumb T, Takeichi M, Ikura M. 1994. Purification and spectroscopic characterization of a recombinant amino-terminal polypeptide fragment of mouse epithelial cadherin. FEBS Lett 352:318–322.

Toole BP. 1972. Hyaluronate turnover during chondrogenesis in the developing chick limb and axial skeleton. Dev Biol 29:321–329.

Toole BP, Jackson G, Gross J. 1972. Hyaluronate in morphogenesis: Inhibition of chondrogenesis in vitro. Proc Natl Acad Sci USA 69:1384–1386.

Toole BP, Munaim SI, Welles S, Knudson CB. 1989. Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. Ciba Found Symp 143:138–145; Discussion 145–149, 281–285.

Tufan A, Tuan R. 2001. Wnt regulation of limb mesenchymal chondrogenesis is accompanied by altered N-cadherin-related functions. FASEB J 15:1436–1438.

Upholt WB, Olsen BR. 1991. The active genes of cartilage. In: Hall B, Newman S, editors. Cartilage: Molecular aspects. Boca Raton: CRC Press, Inc.; pp 1–57.

Yagi T, Takeichi M. 2000. Cadherin superfamily genes: Functions, genomic organization, and neurologic diversity. Genes Dev 14:1169–1180.

Yoon Y, Oh C, Kang S, Chun J. 2000. Protein kinase A regulates chondrogenesis of mesenchymal cells at the post-precartilage condensation stage via protein kinase C-alpha signaling. J Bone Miner Res 15:2197–2205.

Zimmermann B, Thies M. 1984. Alterations of lectin binding during chondrogenesis of mouse limb buds. Histochemistry 81:353–361.