

# Differential Function of N-Cadherin and Cadherin-7 in the Control of Embryonic Cell Motility

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**Abstract.** Similar amounts of N-cadherin and cadherin-7, the prototypes of type I and type II cadherin, induced cell-cell adhesion in murine sarcoma 180 transfectants, Ncad-1 and cad7-29, respectively. However, in the initial phase of aggregation, Ncad-1 cells aggregated more rapidly than cad7-29 cells. Isolated Ncad-1 and cad7-29 cells adhered and spread in a similar manner on fibronectin (FN), whereas aggregated cad7-29 cells were more motile and dispersed than aggregated Ncad-1 cells. cad7-29 cells established transient contacts with their neighbors which were stabilized if FN-cell interactions were perturbed. In contrast, Ncad-1 cells remained in close contact when they migrated on FN. Both  $\beta$ -catenin and cadherin were more rapidly down-regulated in cad7-29 than in Ncad-1 cells treated with cycloheximide, suggesting a higher turnover rate for cadherin-7-mediated cell-cell contacts than for those mediated by N-cadherin. The extent of FN-dependent

focal adhesion kinase phosphorylation was much lower if the cells had initiated N-cadherin-mediated rather than cadherin-7-mediated cell adhesion before plating. On grafting into the embryo, Ncad-1 cells did not migrate and remained at or close to the graft site, even after 48 h, whereas grafted cad7-29 cells dispersed efficiently into embryonic structures. Thus, the adhesive phenotype of cadherin-7-expressing cells is regulated by the nature of the extracellular matrix environment which also controls the migratory behavior of the cells. In addition, adhesions mediated by different cadherins differentially regulate FN-dependent signaling. The transient contacts specifically observed in cadherin-7-expressing cells may also be important in the control of cell motility.

**Key words:** fibronectin • cadherins • transient adhesion • cell motility • embryogenesis

**I**NTERACTIONS of cells with their environment play a key role during embryogenesis. They contribute to important morphological and biological processes in histogenesis and differentiation. These interactions are partly mediated by cell surface receptors and extracellular components belonging to large families. Cadherins and integrins are the major classes of cell surface receptor mediating cell-cell and cell-matrix adhesion, respectively (Hynes, 1992; Takeichi, 1995; Gumbiner, 1996). Based on structural criteria, classical cadherins are classified into two subfamilies, type I and type II cadherins. They are spatio-temporally regulated during embryonic development in a manner that suggests they play a major role in

the control of tissue segregation and in the maintenance of tissue integrity by stabilizing intercellular adhesions. Specific cadherin subtypes are also responsible for transient cell-cell adhesion in the mesenchyme. For example, cadherin-11 is produced by all mesenchymal cells involved in various morphogenetic events, such as during lung and kidney branching morphogenesis, during epithelio-mesenchymal transition (EMT)<sup>1</sup> during sclerotomal formation, by cells undergoing condensation during chondrogenesis, and by migratory cells derived from the neural crest (NCC) (Hoffmann and Balling, 1995; Kimura et al., 1995; Simonneau et al., 1995; Vallin et al., 1998). Development of the NCC is a major morphogenetic process involving various biological steps such as EMT, cell migration, cell aggregation, and cell differentiation (Le Douarin, 1982). All of these processes are associated with a strictly regulated pattern of cadherin synthesis. Premigra-

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1. *Abbreviations used in this paper:* CHX, cycloheximide; ECM, extracellular matrix; EMT, epithelio-mesenchymal transition; FAK, focal adhesion kinase; FN, fibronectin; NCC, neural crest cell.

tory NCC produce N-cadherin and cadherin-6B (Nakagawa and Takeichi, 1995, 1998). However, both are strictly downregulated when these cells separate from the neural tube and start to produce cadherin-7. When NCC reaggregate and differentiate at specific sites, they start producing N-cadherin again (Hatta et al., 1987; Duband et al., 1988; Nakagawa and Takeichi, 1995, 1998). The regulation of cell-matrix adhesion molecules during these morphological processes has been described (Delannet and Duband, 1992; for review see Duband et al., 1995). Extracellular matrix (ECM) components are also spatio-temporally regulated in the NCC environment. For example, fibronectin (FN) and type I collagen are particularly abundant along the migratory pathways of NCC, whereas laminin and type IV collagen are found in the basal surfaces of epithelia but are rarely associated with NCC. At sites of NCC aggregation resulting in ganglion formation, FN and laminin are oppositely regulated: FN synthesis decreases and NCC begin to synthesize laminin (Newgreen and Thiery, 1980; Duband and Thiery, 1987). In addition, competitive inhibitors of cell-matrix interactions block NCC migration (Boucaut et al., 1984; Bronner-Fraser, 1985). The coordinated regulation of cell-cell and cell-matrix adhesion, in addition to providing attractive and repulsive cues (Krull et al., 1997; Wang and Anderson, 1997), is probably also involved in the migration of NCC but the molecular mechanisms involved are poorly understood.

In recent years, the molecular basis for cell adhesion has been extensively investigated. In particular, a major effort has been made to elucidate the signaling events regulating cell-matrix adhesion. These signaling pathways have features in common with those triggered by other cell surface receptors (for reviews see Yamada and Miyamoto, 1995; Yamada and Geiger, 1997; Howe et al., 1998). Numerous studies suggest that there is a cross-talk between cell-cell and cell-matrix adhesion molecules (Hodivala and Watt, 1994; Monier-Gavelle and Duband, 1997; Lu et al., 1998), but little is known about cadherin-mediated signaling cascades. Recently, it has been shown that selective regulation of cell-cell and cell-matrix interactions mediated by integrins or cadherin ligands depends on tyrosine phosphorylation (Levenberg et al., 1998). In addition, integrins and cadherins act in synergy to regulate the contact-mediated inhibition of cell motility in myoblasts (Huttenlocher et al., 1998).

In vitro, adhesion assays have shown that both type I and type II cadherins mediate cell-cell adhesion. However, their different patterns of synthesis in the embryo suggest different roles for these two types of cadherin in EMT and cell migration (Hatta et al., 1988; Nakagawa and Takeichi, 1995). N-Cadherin and E-cadherin, prototype type I cadherins, seem to be involved mainly in the formation and maintenance of cohesive tissues and they interfere strongly with cell motility. It has also been shown recently that the juxtamembrane domain of E-cadherin plays a key role in the inhibition of cell motility in vitro (Chen et al., 1997). In contrast, type II cadherins such as cadherin-7 and cadherin-11 are synthesized by migratory cells. One way to analyze the respective roles of type I and type II cadherins in the control of cell behavior and motility is to compare their effects in in vitro and in vivo systems. We have previously developed an in vivo assay of cell motility. This

model system is based on the ability of S180 cells to migrate if introduced into the NCC migratory pathway of chicken embryos. It has been successfully used to demonstrate that changes in the pattern of integrin expression modify migratory cell behavior both in vitro and in vivo (Beauvais et al., 1995).

In this study, we analyze the in vitro and in vivo behavior of transfected S180 cells producing either chicken N-cadherin or cadherin-7. Our results demonstrate that, depending on the type of cadherin expressed at the surface, cells differentially migrate to ECM components, especially to FN. This phenomenon is even more prominent in vivo than in vitro. Transient contacts in cadherin-7-expressing cells may be an important event controlling cell motility, whereas stable contacts mediated by N-cadherin interfere with cell migration. FN-dependent focal adhesion kinase (FAK) phosphorylation was reduced in cells already engaged in cell-cell adhesion, which is more prominent in N-cadherin-expressing cells. These data suggest that different cadherin types regulate cell adhesion and migration depending on the ECM environment, probably by differential regulation of FN-dependent signaling.

## Materials and Methods

### Antibodies and Reagents

FN, laminin I, vitronectin, type I and type IV collagens, ECM gel, FITC-phalloidin, RGDS peptide, control rat IgG, the mouse mAb (clone GC-4) directed against the A-cell adhesion molecule (A-CAM, also known as N-cadherin), and the rabbit polyclonal anti-pan-cadherin antibody were purchased from Sigma Chemical Co. Mouse mAbs directed against  $\beta$ -catenin (clone 14) and against FAK (clone 77) were obtained from Transduction Laboratories. The mouse mAb directed against phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnologies. The rat mAb directed against mouse  $\beta$ 1 integrin (clone 9EG7) was purchased from PharMingen. The NC-1 mouse mAb against avian NCC has been previously described elsewhere (Vincent et al., 1983). The mouse mAb (clone F-VII) against human vinculin was generously donated by M. Glukhova (Institut Curie, France). The rat mAb NCD-2 and the mouse mAb CCD7-1 were kindly donated by M. Takeichi and S. Nakagawa (Kyoto University, Kyoto, Japan) and are directed against chicken N-cadherin and cadherin-7, respectively. Secondary antibodies coupled to Texas red and horseradish peroxidase were purchased from Amersham.

### Cell Cultures

The S180 cells used in this study were a subclone derived by Dr. K. Yamada (National Institutes of Health, Bethesda, MD) from the original parental cell line, which was obtained from American Type Culture Collection. These cells were selected for their inability to assemble a FN matrix at the cell surface. They were cultured in DMEM containing 10% (vol/vol) FCS, penicillin (100 IU/ml)/streptomycin (100  $\mu$ g/ml), and 2 mM L-glutamine (Seromed) in a 37°C incubator under an atmosphere of 6% CO<sub>2</sub>/94% air. The Ncad-1 expressor clone for chicken N-cadherin and the cad7-29 expressor clone for chicken cadherin-7 were kindly produced by S. Nakagawa. In brief, S180 cells were transfected with either pMiwCad7 (Nakagawa and Takeichi, 1995) or pMiwN (Fujimori and Takeichi, 1993) together with pGKNeoB by the calcium-phosphate precipitation method. Transfectants were selected in culture medium containing G418 (Life Technologies).

### Cell-FN Adhesion Assay

Assays of cell adhesion to FN-coated substrates were performed on bacteriological petri dishes. Droplets of FN (100  $\mu$ l of 0.01–50  $\mu$ g/ml in PBS containing 10  $\mu$ g/ml BSA) were deposited on bacteriological dishes and incubated overnight at 4°C followed by a 30-min incubation with 3 mg/ml BSA in PBS (previously heat-inactivated for 3 min at 80°C). The substrates were thoroughly washed and maintained in PBS until use. S180,

cad7-29, and Ncad-1 cells were harvested with cell dissociation enzyme-free buffer (Life Technologies) for 10 min at 37°C. Cells were pelleted by centrifugation and incubated for 45 min in DMEM containing with 10% (vol/vol) FN-depleted FCS + 1 mM EGTA to prevent self cell-cell aggregation. Cells were then centrifuged, resuspended at a density of  $2.5 \times 10^5$  cells/ml, and 100- $\mu$ l droplets of the suspension were deposited on the pre-coated substrates. The dishes were incubated at 37°C for 1 h, rinsed with PBS to remove the nonadherent cells, and fixed in 5% glutaraldehyde in PBS. Cells were observed and images were recorded using a Nikon phase-contrast microscope equipped with a high performance CCD camera. The images were transferred to a Power Macintosh G3 computer. Cell adhesion was then quantified by colorimetric analysis. Cells were stained with 1% crystal violet in 200 mM MES and destained in 200 mM MES buffer, pH 6. The crystal violet fixed to the cells was dissolved in 10% acetic acid and OD was measured at 570 nm. The control values correspond to the OD obtained for cells deposited on BSA. 100% cell adhesion corresponds to the value obtained on FN-coated substrate at 50  $\mu$ g/ml. The extent of cell spreading was quantified using Scion Image software by measuring the area of cells interacting with the substrate (at least 30 cells were measured). At least three independent experiments were done for each FN concentration and for each cell type.

### Cell-Cell Adhesion Assay

S180, cad7-29, and Ncad-1 cells were plated in culture 1 d before the assay. They were harvested from monolayer cultures as previously described (Nakagawa and Takeichi, 1995) to preserve cadherins at the cell surface.  $2 \times 10^6$  cells were added to an Erlenmeyer flask containing 5 ml of HMF (10 mM Hepes, magnesium-free PBS, pH 7.4, 10 mM  $\text{CaCl}_2$ ). The cell suspensions were incubated at 37°C in a gyratory shaker at 75 rpm. The kinetics of cell aggregation were determined by collecting several aliquots of the cell suspension at various times. The degree of cell aggregation was estimated according to the decrease in particle number:  $\text{percent aggregation} = 1 - [\text{number of particles } P_x \text{ (at time } t = x)] / \text{initial particle number } P_0] \times 100$ . At least three independent experiments were done for S180, cad7-29, and Ncad-1 cells.

### Labeling of Cells

Before grafting experiments or aggregation assay, cells were labeled with fluorogold as previously described (Beauvais et al., 1995). In brief, 50% confluent cell cultures were incubated for 4 h in 4 ml of culture medium containing 24  $\mu$ l of fluorogold stock solution (2% in Locke's saline; Fluorochrome Inc.). The cells were washed three times in Hanks' balanced salt solution (Life Technologies) to remove all residual label. Cells were harvested in a solution of trypsin/EDTA, centrifuged, and resuspended in fresh culture medium.

### Aggregate Dispersion Assay

$5 \times 10^6$  cells were added to an Erlenmeyer flask containing 5 ml of DMEM supplemented with 10% (vol/vol) FCS, previously equilibrated in a 6%  $\text{CO}_2/94\%$  air atmosphere. The cell suspensions were incubated at 37°C in a gyratory shaker at 75 rpm for 24 h.

Cell aggregates were dispersed on bacteriological petri dishes or glass coverslips coated with 100- $\mu$ l droplets of FN (10  $\mu$ g/ml), laminin (50  $\mu$ g/ml), type I collagen (30  $\mu$ g/ml), type IV collagen (30  $\mu$ g/ml), or vitronectin (10  $\mu$ g/ml) in PBS and were incubated at 37°C for 1 h. Coated substrates were rinsed twice in PBS, saturated with BSA, and extensively washed. Aggregates were then deposited and allowed to disperse before fixation.

Aggregates were cultured in a three-dimensional environment, by allowing a mixture of one part ECM gel to one part DMEM (vol/vol) containing a few aggregates to gel at 37°C for 30 min before incubation with the culture medium.

For the mixed cell aggregation assay, Ncad-1 cells and fluorogold-labeled cad7-29 cells were mixed in an Erlenmeyer flask in a 1:1 ratio at a total cell density of  $5 \times 10^6$  cells/ml. They were incubated in a gyratory shaker at 37°C for 16 h. Aggregates were fixed by incubation with 0.5% glutaraldehyde for 5 min, plated on glass coverslips, and observed by fluorescence microscopy.

The dispersion of aggregates was observed at various times with a Nikon inverted phase-contrast microscope. In some experiments, dispersing aggregates were incubated for 1 h with 100  $\mu$ g/ml of inhibitory rat monoclonal anti-mouse  $\beta 1$  integrin, control rat IgG, or 2 mg/ml RGDS peptides with or without cycloheximide (CHX, 10  $\mu$ g/ml) before fixation.

### Time-Lapse Videomicroscopy

Cell aggregates were deposited on an FN-coated bacteriological petri dish and incubated at 37°C in culture medium. The plate was then placed on the stage of a computer-controlled epifluorescence microscope (Leica DMIRBE) equipped with an enclosed warming incubator, a cooled CCD camera (Princeton RTE/CCD), and a PC workstation. The enclosed incubator made it possible to maintain the sample at 37°C in a humidified atmosphere containing 6%  $\text{CO}_2/94\%$  air. Metamorph software (Universal Imaging) controlled image acquisition, light intensity, fluorescence shutter, filter wheel, and motorized stage. Images were recorded every 4 min. Migratory cell paths were traced and the speed of locomotion was calculated by dividing the total distance of migration by the time of the experiment. This was done for at least 20 cells in two independent experiments.

### The Varani Assay for Cell Migration

Cell migration was quantified by determining the extent of migration from agarose droplets using a modification of the method described by Varani et al. (1978). In brief, cells were harvested by trypsin-EDTA treatment and centrifugation and were resuspended at a density of  $3.3 \times 10^7$  cells/ml in  $\text{CO}_2$ -independent medium (Life Technologies) containing 0.2% low-melting agarose (Sea Plaque, FMC Bioproducts, TEBU) maintained at 39°C. The cells were plated as 0.8- $\mu$ l droplets on flat-bottomed wells (microtest III flexible 96-well assay plates, non-tissue culture-treated; Falcon) precoated with 10  $\mu$ g/ml FN and the agarose was allowed to solidify for a few minutes at 4°C. Culture medium was added with care to prevent the detachment of droplets. Cells were cultured at 37°C for 20 h and fixed with 3.7% formaldehyde. Cells were rinsed, stained with crystal violet, and rinsed again. Images were recorded using a Nikon phase-contrast microscope equipped with a high performance CCD camera. They were transferred to a Power Macintosh G3 computer. The total area of cell outgrowth was measured directly on the monitor for at least 24 droplets for each cell type. The results are expressed in arbitrary units.

### Immunofluorescence Microscopy of Cells

Cells were rinsed twice in PBS containing 2 mM  $\text{CaCl}_2$  and  $\text{MgCl}_2$  and incubated in methanol for 10 min at  $-20^\circ\text{C}$ . They were then incubated with acetone for 2 min and air dried. The cells were rehydrated in PBS and treated with blocking solution (3% BSA in PBS) for 30 min. They were then stained for 1 h at room temperature with primary antibody diluted in blocking solution. Cells were rinsed twice in PBS and incubated with secondary antibody coupled to Texas red for 45 min. Before immunofluorescent labeling with FITC-phalloidin or antivinculin antibody, the cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeabilized by incubation for 5 min in 0.5% Triton X-100 in PBS, rinsed three times, incubated for 30 min in 20 mM glycine in PBS, and treated with blocking solution (3% BSA in PBS) for 30 min to reduce background signal. Preparations were observed using a motorized epifluorescence microscope (Leica DMRBE) equipped with a cooled CCD camera (Hamamatsu C5985). Acquisitions were controlled by a Power Macintosh workstation via IP-Lab software. Images were recorded at the same time as exposure and gain values to make it possible to compare in fluorescence intensity.

### Western Blot Analysis

For Western blot analysis of  $\beta$ -catenin or cadherins, subconfluent monolayers of cells were grown in standard conditions or in culture medium containing 10  $\mu$ g/ml of CHX for 2–5 h before extraction. Cell extracts were prepared in situ by incubation with 1 ml/petri dish of lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM PMSF, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin in PBS containing 2 mM  $\text{CaCl}_2$  and  $\text{MgCl}_2$ ) for 15 min on ice.

For Western blot analysis of tyrosine-phosphorylated proteins, cell extracts were prepared in situ by incubation with lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 2 mM PMSF, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, 100  $\mu$ M sodium orthovanadate) for 15 min on ice. Four different treatments were applied to cells before extraction. Cells were harvested as described for the cell-cell adhesion assay and were allowed to aggregate in HMF for 2 h to produce well defined aggregates (except for S180 cells which cannot aggregate). They were centrifuged before extraction (lane 1 of Western blot). Alternatively, cells were incubated in suspension for 10 min (Ncad-1) or 30 min (cad7-29 and S180) to ensure that most of the cells were engaged in the initial intercellular aggregation process (small aggregates, except for S180 cells which cannot ag-

gregate). Cells were then plated for 30 min on FN- (10  $\mu$ g/ml; lane 3) or BSA-coated substrate (lane 4) before extraction. Alternatively, cells were harvested as described for the cell-FN adhesion assay and plated for 30 min on FN-coated substrate (at a density preventing cell-cell adhesion) before cell extraction (lane 2).

Cells were incubated with lysis buffer on ice, scraped from the petri dish with a rubber policeman, homogenized, and centrifuged for 15 min at 20,000 *g* at 4°C. Supernatants were collected and protein content determined by protein assay (Bio-Rad). Proteins (50  $\mu$ g in SDS sample buffer) were subjected to electrophoresis in 7.5% or 10% acrylamide gels. Proteins were transferred electrophoretically from gels to Immobilon-P filters (Millipore). The membranes were incubated with appropriate antibodies. In brief, membranes were incubated with blocking solution (0.1% gelatin, 0.1% Tween 20 in PBS) for 1 h on a gyratory shaker at room temperature and were rinsed four times in PBS containing 0.1% Tween 20. They were incubated overnight at 4°C with anti- $\beta$ -catenin (1:2,000), CCD7.1 ascites (1:4,000), anti-pan-cadherin serum (1:2,000), anti-FAK (1:1,000), or anti-phosphotyrosine (4G10; 1:1,000) in GT-PBS. Membranes were thoroughly washed and incubated for 1 h with anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase at a dilution of 1:10,000. Membranes were washed and incubated with a chemiluminescence detection reagent (Amersham) for 1 min. The reagent was drained off and the membranes were placed against Hyperfilm (Amersham).

### Grafting Experiments

The Ncad-1 and cad7-29 cells were labeled with fluorogold, treated with trypsin-EDTA solution, centrifuged, and allowed to form small aggregates for 4 h. The aggregates were rinsed in sterile Locke's saline medium (LSM). They were then transferred to a petri dish containing LSM, and were lightly stained with a few drops of 0.2% neutral red.

The parental S180 cells were labeled with fluorogold, treated with trypsin-EDTA solution, and centrifuged. The cell pellet was resuspended in fresh culture medium and incubated for 2 h in a 37°C water bath to allow the cells to reconstitute membrane proteins. The cells were repelleted by centrifugation. The pellet was transferred to a petri dish containing LSM, and was lightly stained with a few drops of 0.2% neutral red.

White Leghorn chicken embryos were incubated at 38°C until they had developed 20–23 somite pairs, corresponding to stage 13–14 (according to Hamburger and Hamilton, 1951). Grafting experiments were performed as previously described (Beauvais et al., 1995). A small aggregate or cell pellet was inserted into the embryo with a Spemann pipette. It was gently maneuvered with a tungsten needle through a slit in the ectoderm between the neural tube and somite in the area to which NCC migrate after leaving the dorsal surface of the neural tube (i.e., the seventh somites anterior to the last-formed somite; according to Loring and Erickson, 1987). The relocation of the graft site was facilitated by inserting a small amount of black charcoal into the center of the second somite posterior to the graft site. The embryo was then carefully picked up with a ring of filter paper and suspended on a nucleopore filter (polycarbonate, 8- $\mu$ m pores) over the well of an organ culture dish (Falcon) filled with L15 medium (Life Technologies) containing 10% FCS. The embryos were returned to the 38°C incubator for a further 18 h and were then fixed. Grafts for 48-h grafting experiments were done in a similar manner, but directly in ovo after opening the shell with scissors and making a hole in the vitelline membrane as described by Selleck (1996).

### Histology

Embryos were fixed in 4% paraformaldehyde for 2 h, washed with PBS, and a region of the embryo six somites in length, encompassing the graft, was cut out with tungsten needles. Embryo pieces were washed for 3 h in blocking solution and incubated overnight at 4°C in 0.5% BSA in PBS containing NC-1 antibody to label the NCC. Unbound antibody was removed by a 3-h incubation in 0.5% BSA in PBS and the specimens were immersed overnight in the secondary antibody. Specimens were rinsed several times and postfixed by incubation in 0.1% paraformaldehyde for 1 h and rinsed twice in PBS. The explants were then dehydrated, incubated twice for 3 min each in xylene, embedded in paraplast, and sectioned as previously described (Levi et al., 1987).

### Results

N-Cadherin and cadherin-7 belong to different cadherin subfamilies, type I and II, respectively. They are specifi-

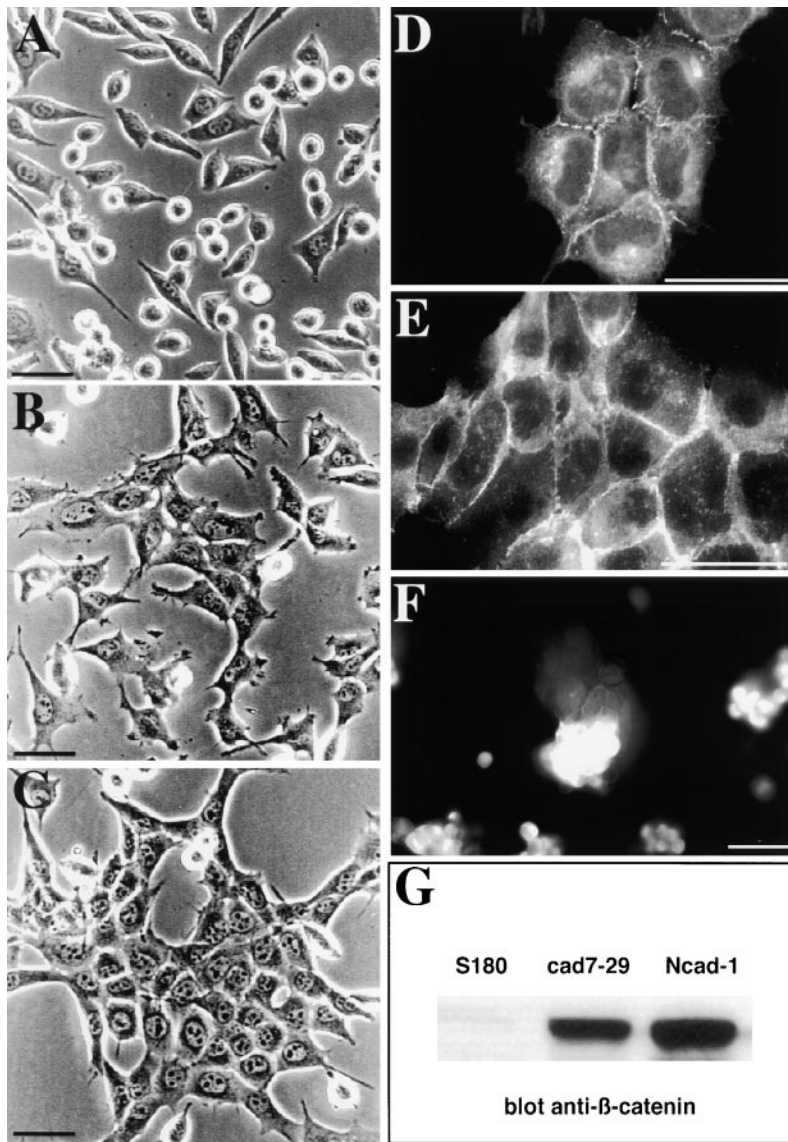
cally regulated during NCC migration. N-Cadherin is expressed by NCC resident in the neuroepithelium and after aggregation to form ganglia, whereas cadherin-7 starts to be synthesized only when NCC begin to migrate. The spatio-temporal distribution of these cadherin subtypes during early embryogenesis suggests different functions in cell migration. We investigated the role of these cadherins in the control of *in vitro* and *in vivo* cell motility using S180 cells genetically modified by stable transfection so as to synthesize chicken N-cadherin or cadherin-7. S180 cells were used because they have a fibroblast-like morphology and no cell-cell adhesion properties. These cells are able to migrate after grafting into the embryonic environment (Erickson et al., 1980). They are a good model system for investigating the role of cadherins in the control of cell motility in the embryo.

### Adhesion Properties of Cadherin-expressing Cells

Stable S180 transfectants expressing cadherin-7 (Fig. 1 B) or N-cadherin (Fig. 1 C) exhibited morphological differences from parental S180 cells (Fig. 1 A) characteristic of a cell-cell adhesive phenotype. The two types of transfectants were more flattened than parental cells. Ncad-1 cells gave rise to clusters of cells that were more cohesive than those of cad7-29 cells. Cadherin-7 (Fig. 1 D) and N-cadherin (Fig. 1 E) are present along cell boundaries, as shown by fluorescence immunostaining with specific antibodies CCD7-1 and NCD2, respectively.

Ncad-1 and cad7-29 cells, harvested from tissue culture dishes and put into suspension, form aggregates. Fluorogold-labeled cad7-29 cells were mixed with unlabeled Ncad-1 cells and allowed to aggregate for 16 h. Each clone segregated to form separate cohesive aggregates that could be distinguished under UV illumination (Fig. 1 F). Unlabeled aggregates were sometimes found apposed to labeled aggregates but no mixed aggregates were formed. This phenomenon is typical of a homotypic aggregation and shows that N-cadherin cannot interact with cadherin-7 as previously observed for L cells (Nakagawa and Takeichi, 1995). We analyzed cadherin expression in each transfectant by Western blot analysis of  $\beta$ -catenin content. We did this to determine whether  $\beta$ -catenin was similarly up-regulated relative to the endogenous level of  $\beta$ -catenin in parental cells (Fig. 1 G). S180 cells had very low levels of  $\beta$ -catenin which was only detectable on overexposed autoradiographs. The expression of either cadherin-7 or N-cadherin induced a strong increase in the amount of  $\beta$ -catenin. We performed several Western blot analyses and observed similar or slightly higher levels of  $\beta$ -catenin in Ncad-1 cells (see also Fig. 9). This suggests that cad7-29 and Ncad-1 cells have similar amounts of cadherin-7 and N-cadherin, respectively, at the cell surface.

Cell-cell adhesion assays were performed to compare the intercellular adhesion properties of parental and transfectant cells (Fig. 2). During the initial phase of the aggregation process, a major difference was observed between Ncad-1 and cad7-29 cells. 70% of cell adhesion was achieved by Ncad-1 cells within the first 15 min, whereas the same extent of cell aggregation took 1 h to achieve for cad7-29 cells (Fig. 2 A). Ncad-1 and cad7-29 cells reached their maximum level of cell adhesion after  $\sim$ 4 h, whereas



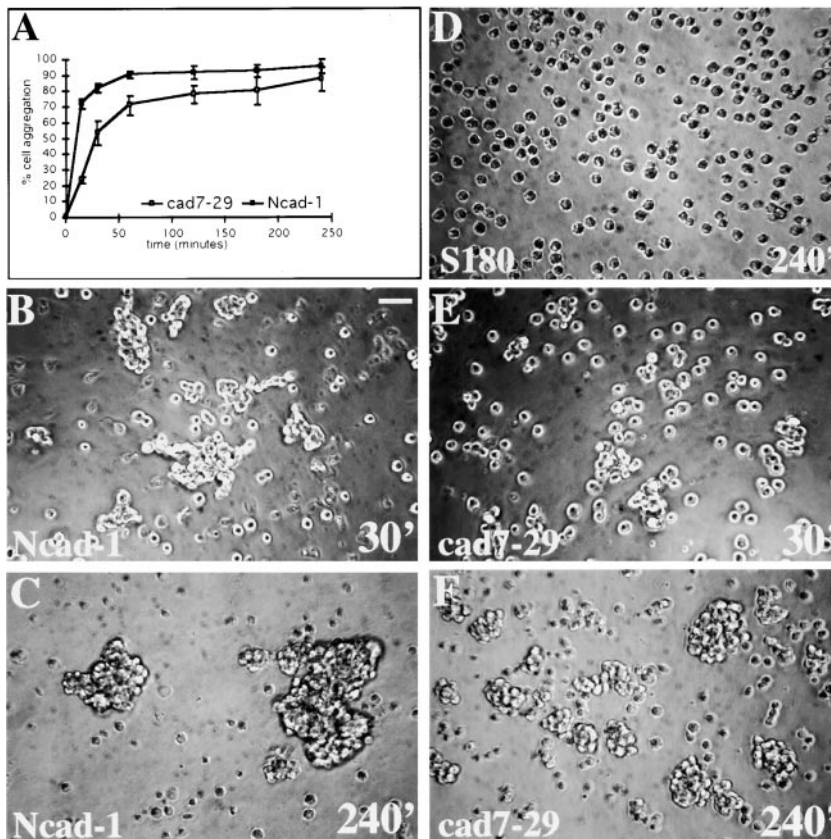
**Figure 1.** Adhesive properties of transfected cells. Morphology of parental S180 cells (A), cad7-29 (B), and Ncad-1 cells (C) on tissue culture dishes. Cadherin-7 (D) and N-cadherin (E) are present at cell boundaries of the corresponding cells. After mixing unlabeled Ncad-1 cells and fluorogold-labeled cad7-29 cells in suspension, homotypic aggregates were observed (F, under UV light). Note the almost complete segregation of Ncad-1 and cad7-29 cell transfectants. (G) Western blot for  $\beta$ -catenin in 50  $\mu$ g of cell protein extract obtained from S180 cells and the two transfectants. Note that much more  $\beta$ -catenin is present in cad7-29 and Ncad-1 cells than in parental S180 cells. Bars, 50  $\mu$ m.

parental cells did not aggregate (Fig. 2 D). At that time and earlier during the aggregation process, Ncad-1 cells formed larger aggregates (Fig. 2, B and C) than cad7-29 cells (Fig. 2, E and F). Cell aggregates continued to grow in size thereafter but after 24 h there is a little difference between Ncad-1 and cad7-29 aggregate sizes (see Fig. 3, A and F).

#### **Differential Dispersion of cad7-29 and Ncad-1 Aggregates on Coated Substrates**

We investigated the response of transfected cells to the ECM environment by analyzing the ability of cad7-29 (Fig. 3, A-E, and Fig. 4 A) and Ncad-1 aggregates (Fig. 3, F-J, and Fig. 4 C) to spread and disperse in vitro on two-dimensional substrates coated with FN, laminin-1, type I collagen, type IV collagen, or vitronectin and within a three-dimensional ECM gel (composed mostly of laminin-1, type IV collagen, nidogen, and heparan sulfate proteoglycans).

cad7-29 aggregates were totally dispersed on FN after 5 h (Fig. 3 B). They were less efficiently dispersed on laminin-1 (Fig. 3 C) and on vitronectin (Fig. 3 D). Ncad-1 aggregates also dispersed on FN (Fig. 3 G) and on laminin-1 (Fig. 3 H) but to a lesser extent than observed for cad7-29 aggregates, whereas dispersion on vitronectin was inefficient (Fig. 3 I). After 5 h of culture, aggregates of both cad7-29 (Fig. 4 A) and Ncad-1 (Fig. 4 C) adhered but could not disperse on type I collagen. In contrast, the two types of aggregates did not adhere to type IV collagen within the first 5 h of culture (not shown). However, isolated S180, Ncad-1, and cad7-29 cells adhere to both type I and type IV collagen-coated substrates within an hour, but could not spread on them in standard cell-substrate adhesion assays (not shown). Thus, the greatest difference in scattering between Ncad-1 and cad7-29 aggregates was observed on FN. Ncad-1 cells migrating away from aggregates were tightly apposed to each other, whereas cad7-29 cells were more loosely connected and were sometimes found as individual cells.



**Figure 2.** Intercellular adhesion properties of cells. In A, cell-cell adhesion kinetics of Ncad-1 (black squares) and cad7-29 (white squares) cells over 4 h in HMF. Almost 70% cell adhesion was observed after 15 min for Ncad-1 and after 1 h for cad7-29. After 4 h, maximal cell adhesion was achieved for both cell types. Data are expressed as mean percent cell aggregation  $\pm$  the standard error calculated from three independent experiments. Ncad-1 cells (B and C) formed larger aggregates than cad7-29 cells (E and F). In contrast, parental S180 cells (D) displayed no cell adhesion properties in these conditions. Bar, 50  $\mu$ m.

We quantified the dispersion of Ncad-1 and cad7-29 aggregates by measuring the area of 10 aggregate outgrowths at various times ( $t = x$ ) and calculating the mean area ( $O$ ). The ratio  $O(t = x)/O(t = 0)$  was calculated for the two types of aggregate deposited on each coated substrate (Fig. 4, B and D). Over a 210-min period, the two types of aggregate disseminated in a similar manner on laminin-1 and vitronectin. Thereafter, cad7-29 aggregates dispersed more rapidly than Ncad-1 aggregates on vitronectin. In contrast, they did not disperse on type I collagen during this period. On FN, a significant difference in scattering was observed between the two types of aggregate, both in the timing of dispersion and its extent. cad7-29 cells started to escape from the aggregate within 30 min, whereas it took  $>90$  min for Ncad-1 cells to emerge. This difference in dispersion increased rapidly with time and after 5 h the ratio  $O(t = x)/O(t = 0)$  for dispersing cad7-29 aggregates was three times higher than that obtained for Ncad-1.

We assessed the ability of cell aggregates to disperse in a three-dimensional environment by analyzing their behavior inside an ECM gel. No invasion was observed within 5 h (data not shown). However, after 24 h both types of aggregate invaded the ECM gel (Fig. 3, E and J) with cad7-29 cells invading more efficiently. In this environment, cad7-29 cells appeared to be connected with only a few other cells and formed aligned cell structures migrating into the ECM gel. Ncad-1 cells did escape from the aggregates, but as strongly cohesive sheets of cells.

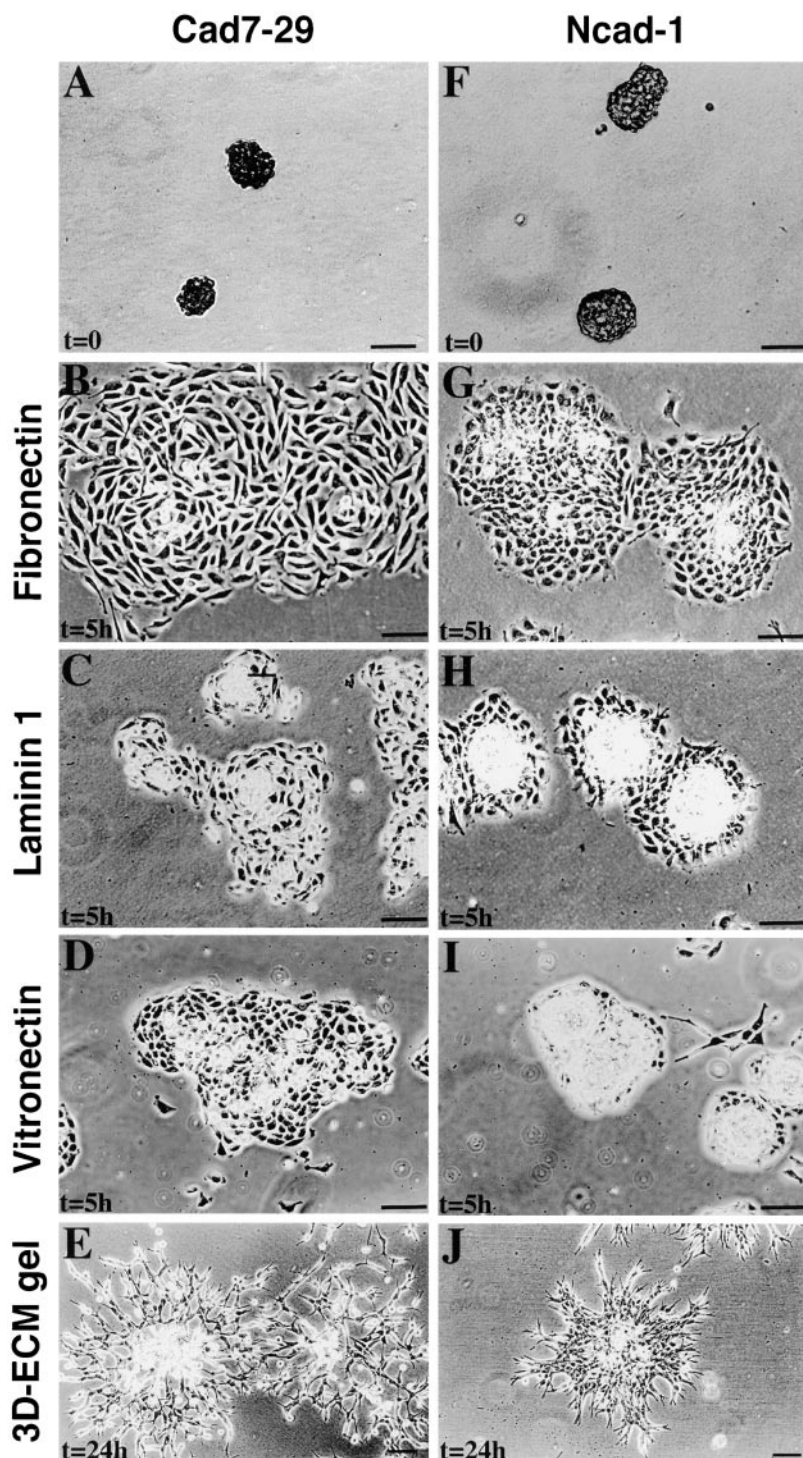
One explanation for the differences in dispersion behav-

ior of cad7-29 and Ncad-1 cell aggregates is that these cells have a different pattern of integrins at their surface. We explored this possibility by immunoprecipitation experiments with specific antibodies against integrins; both transfectants had the same integrin pattern as that previously described for parental S180 cells (Beauvais et al., 1995; large amounts of  $\alpha 3\beta 1$ ,  $\alpha \nu \beta 1$ , and  $\alpha \nu \beta 3$  integrins, small amounts of  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$ ). A slight increase was found in the amount of  $\alpha 6\beta 1$  integrin for Ncad-1 cells (not shown).

#### **cad7-29 Cells Exhibit a Higher Migratory Capability Than S180 and Ncad-1 Cells**

Time-lapse videomicroscopy was carried out to analyze the behavior of Ncad-1 (Fig. 5 A) and cad7-29 (Fig. 5 C) cells migrating out of aggregates and to determine their speed of locomotion. The migration of cells was followed until aggregates deposited on FN were totally dispersed. Ncad-1 cells migrating as an epithelial sheet were still strongly apposed to their neighbors after 4 h (Fig. 5 B) and longer (not shown). cad7-29 cells were found in contact with their neighbors at the start of their migratory process but they then began to dissociate and established only transient contacts with their neighbors (Fig. 5 D). They migrated faster than Ncad-1 cells, even when they started to migrate when they were still closely interacting. Locomotion speed was calculated as described in Materials and Methods. We found that cad7-29 cells migrated three times faster than Ncad-1 cells ( $57.2 \pm 14.4$  and  $19.5 \pm 4.7$



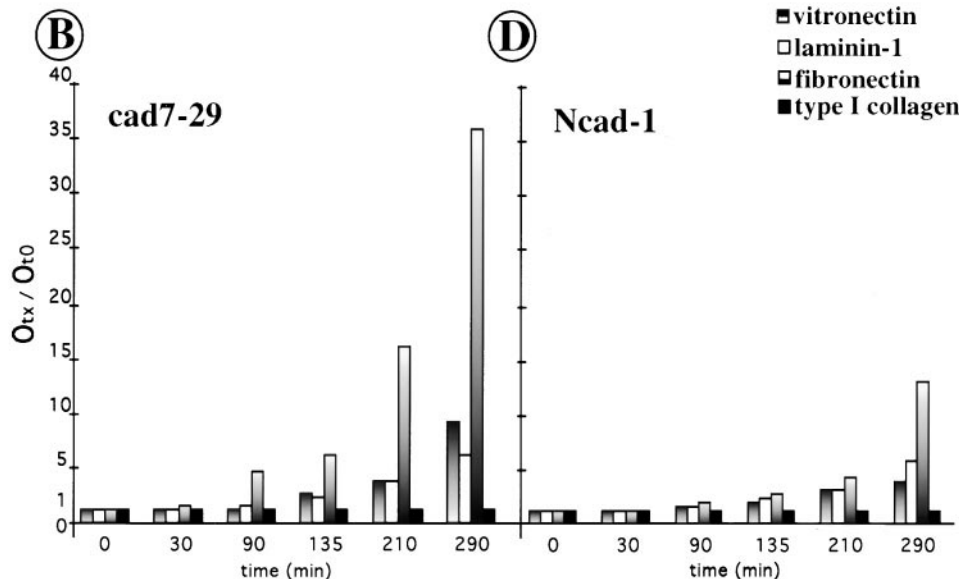
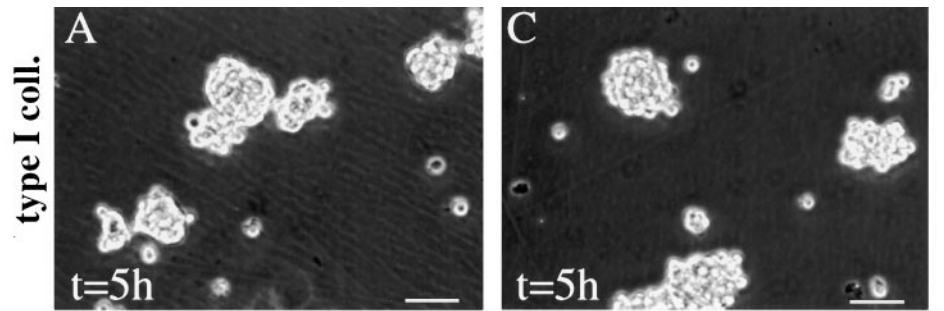


**Figure 3.** Dispersion of cad7-29 (A–E) and Ncad-1 (F–J) aggregates on various coated substrates. The initial sizes of cad7-29 and Ncad-1 aggregates are represented in A and F, respectively. The aggregates were photographed after 5 h of dispersion on FN (B and G), laminin-1 (C and H), and vitronectin (D and I) or after 24 h of culture in a three-dimensional ECM gel (E and J). Bars, 50  $\mu\text{m}$ .

$\mu\text{m}/\text{h}$ , respectively). As the two transfectants displayed similar levels of proliferation (data not shown), their speed of locomotion probably reflects the major difference in their area ratio, calculated after 5 h for aggregate dispersion. It also suggests that, despite high cell density favoring cell-cell contacts inside the outgrowth area, migrating cad7-29 cells gradually became less able to adhere to other cells on FN.

Parental S180 cells migrated on FN with an intermediate speed of locomotion of  $37.4 \pm 12.8 \mu\text{m}/\text{h}$ , but this was

measured for isolated cells because S180 cells does not form aggregate. One possible explanation for the speed of the cad7-29 migration on FN being higher than that of S180 cells is the fact that cad7-29 cells are initially aggregated (Fig. 5 A). A population pressure effect of cad7-29 cells emerging from the aggregate on the cells already migrating on FN could increase the motility of these cells. We analyzed the effect of population pressure on S180 cells by Varani assay. S180, Ncad-1, and cad7-29 were artificially maintained in an aggregated form (independently



**Figure 4.** Dispersion of cad7-29 (A) and Ncad-1 (C) aggregates on type I collagen after 5 h. Quantification of cad7-29 (B) and Ncad-1 (D) aggregate dispersion on various substrates as described in Materials and Methods. We calculated the ratio between  $O_{tx}$  (mean diameter of outgrowth area at time  $t = x$  minutes) and  $O_{t0}$  (mean initial diameter of the aggregate). This was done for at least 10 aggregates cultured on vitronectin (white-black gradual bar), laminin-1 (white bar), FN (black-white gradual bar), and type I collagen (black bar), respectively. Bars in A and C, 50  $\mu\text{m}$ .

of cell adhesion molecules) inside an agarose droplet and deposited on FN substrate. The droplets were cultured for 20 h to allow the cells to escape and migrate from this artificial aggregate. In these conditions, we obtained larger outgrowth areas for cad7-29 cells (Fig. 5 E) than for S180 (Fig. 5 F) or Ncad-1 (Fig. 5 G) cells. Quantification was performed for at least 24 droplets of each cell type (Fig. 5 H) and confirmed the results obtained in videomicroscopy experiments. Thus, in two independent assays we observed that cad7-29 cells migrated faster than S180 and Ncad-1 cells.

Another explanation for these differences in migratory properties is that cad7-29, Ncad-1, and parental S180 cells differentially adhere to or spread on FN. We performed a standard cell-FN adhesion assay in which isolated cells were exposed to various concentrations of FN-coated substrates. We observed that all three cell types adhered and spread in a similar manner to substrates coated with 0.5–50  $\mu\text{g/ml}$  of FN (not shown). We then performed experiments with lower concentrations of FN (0.01–0.5  $\mu\text{g/ml}$ ). Cell adhesion to FN (Fig. 6 A) occurred between 0.01 and 0.05  $\mu\text{g/ml}$ . S180 cells initially seemed to adhere more strongly than cad7-29 and Ncad-1 cells at a concentration of 0.05  $\mu\text{g/ml}$  of FN. However, for higher FN concentrations, there was no obvious difference between the three cell types; we obtained almost 80% cell adhesion at 0.1  $\mu\text{g/ml}$  with maximum of cell adhesion reached at 0.5  $\mu\text{g/ml}$ . Cell

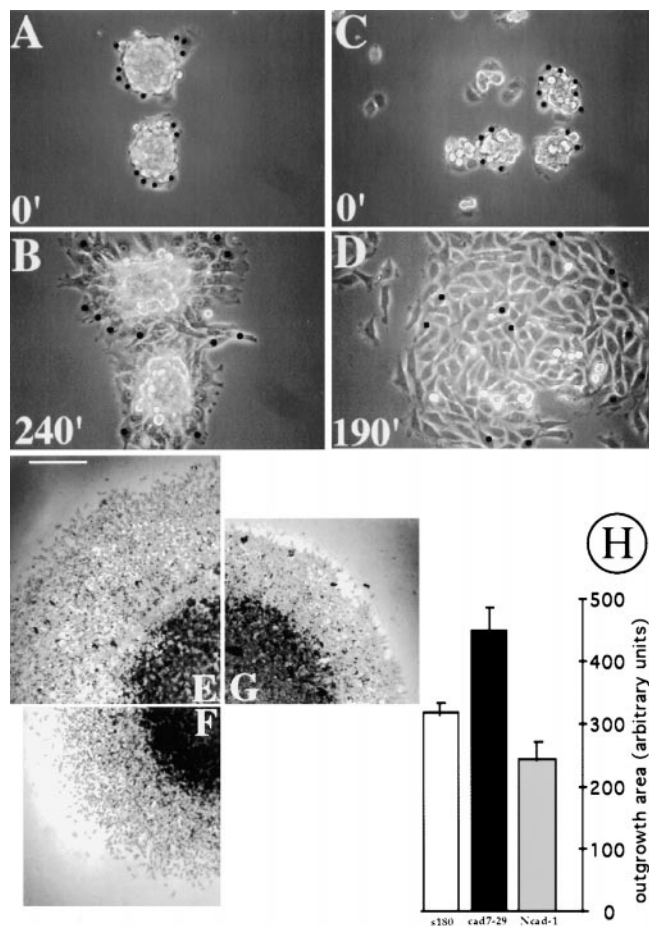
spreading (Fig. 6 B) was calculated by measuring the area of adherent cells for each FN concentration tested. We found that cad7-29 (Fig. 6 D) and Ncad-1 (Fig. 6 E) cells were always more flattened than S180 (Fig. 6 C) cells. This was more evident at a concentration of 0.5  $\mu\text{g/ml}$  which gave maximum cell adhesion.

#### **Cadherins Are Differentially Located in Dispersing Ncad-1 and cad7-29 Cells on FN**

The prevalence of the proteins involved in cell adhesion during aggregate dispersion was analyzed by immunolabeling with specific antibodies directed against N-cadherin, cadherin-7,  $\beta$ -catenin, or vinculin and with phalloidin-FITC which interacts specifically with filamentous actin. Cell aggregates were deposited on glass coverslips with or without a coating of FN and were allowed to scatter for several hours before fixation.

All Ncad-1 cells escaping from aggregates on both uncoated or FN-coated glass coverslips were closely connected to their neighbors. However, Ncad-1 cells appeared to spread more extensively on FN. N-Cadherin (Fig. 7, A and B, top panel),  $\beta$ -catenin (Fig. 7 C, top panel), and  $\alpha$ -catenin (not shown) were strongly detected at cell-cell boundaries. F-Actin was organized as a cortical network along cell-cell contacts and in numerous filaments anchoring both at the tips of lamellipodia (Fig. 7 D, white arrow-





**Figure 5.** The upper panel represents an example of a videomicroscopy experiment involving the dispersal of Ncad-1 and cad7-29 aggregates after plating on FN. Escaping Ncad-1 (A) and cad7-29 (C) cells were followed (black dots) until aggregates were totally dispersed. (B) After 240 min, Ncad-1 aggregates were not totally dispersed, whereas cad7-29 aggregates were (D). The lower panel and the graph show the result of a Varani assay performed over 20 h. As S180 cells do not form aggregates, we produced artificial aggregates of each cell type to compare their migratory properties under population pressure conditions. In brief, small droplets of high-cell density suspension containing 0.2% low-melting agarose were deposited on FN-coated substrate (10  $\mu\text{g/ml}$ ) and an artificial cell aggregate was allowed to form. Droplets were incubated in culture medium for 20 h before fixation. During the incubation, cells escaped from the droplet and migrated on FN. One-quarter images of representative cad7-29, S180, and Ncad-1 outgrowth areas are presented in E–G, respectively. The droplet appears darker than the outgrowth area after crystal violet staining due to the high density of cells inside. Data are expressed as a mean of outgrowth area  $\pm$  the standard error calculated from 24 droplets of S180 (white bar), cad7-29 (black bar), and Ncad-1 (gray bar) cells. Bar in E, 500  $\mu\text{m}$ . F and G are at the same magnification.

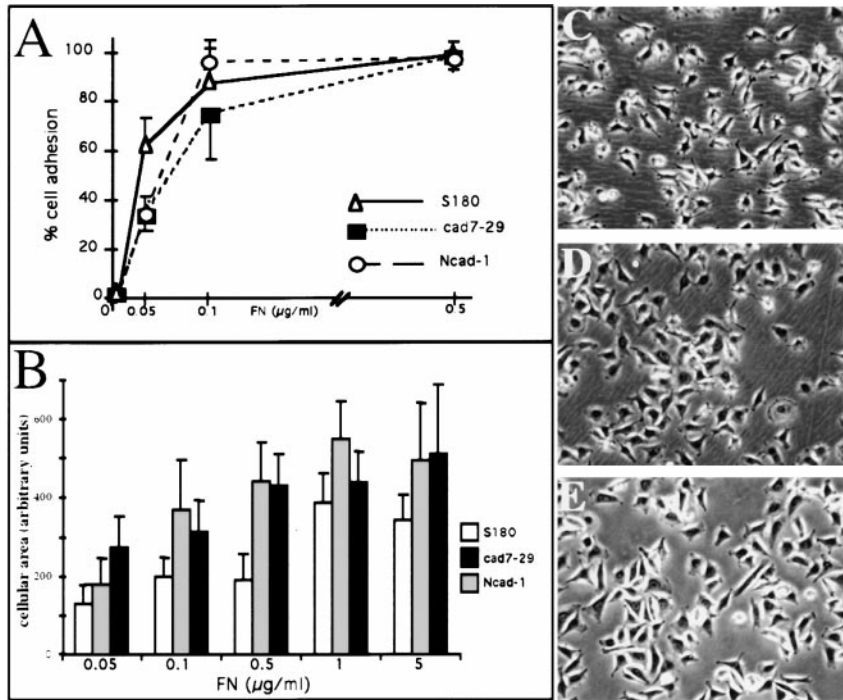
head, top panel) or at cell-cell contacts (adherens junctions; Fig. 7 D, black arrow, top panel). This shows that the locations of N-cadherin-mediated cell-cell contacts are not affected by adhesion to FN.

Cadherin-7 was also strongly located at cell-cell contacts in cad7-29 cells migrating out of aggregates cultured on

glass coverslips (Fig. 7 A, bottom panel). On FN, cad7-29 cells were more flattened and less connected to neighboring cells. The extent of cell-cell contacts for cad7-29 cells and the level of the expression of cadherin (Fig. 7 B, bottom panel),  $\beta$ -catenin (Fig. 7 C, bottom panel), and  $\alpha$ -catenin (not shown) at these sites were lower than those for Ncad-1 cells. Some cells close together produced small amounts of cadherin-7 at cell boundaries. The overall level of cadherin-7 expression appeared lower than that of cells cultured on uncoated coverslips. F-Actin was less organized in cad7-29 cells than in Ncad-1 cells, especially at cell-cell contact sites, where it was found only as a cortical network (Fig. 7 D, black arrow, bottom panel). Fewer actin filaments than in Ncad-1 cells were anchored at cell-cell contacts. Stress fibers were detected at the tips of lamellipodia (Fig. 7 D, white arrowhead, bottom panel) and as small dots along cad7-29 cell-FN contacts. The distribution of vinculin was similar in the two cell types, detected as small dots corresponding to focal sites (not shown). Thus, cadherin-7-mediated cell-cell contacts were regulated differently from those mediated by N-cadherin when cells were interacting with FN.

We further analyzed whether the reduction in the ability of cad7-29 cells to establish cell-cell contacts on FN was reversible. We plated cad7-29 aggregates on this substrate and allowed them to disseminate. We then perturbed cell-FN interactions by incubating cells in the presence of RGDS peptide, inhibitory antibody directed against mouse  $\beta 1$  chain of integrins, or control IgG. We used a concentration of competitors insufficient for complete cell detachment during the assay. Within 1 h of treatment, changes in cell morphology were clearly observed for cad7-29 cells on FN (Fig. 8, top panel). The flattened cell shape observed in the presence of control IgG (Fig. 8 A) was changed in the presence of competitors such as RGDS peptides or anti- $\beta 1$  antibodies (Fig. 8, C and D). Cells were more rounded due to a higher level of cell-cell adhesion. Large amounts of cadherin-7 were present at intercellular contacts. We investigated whether this was due to a redistribution of the protein or to the incorporation of newly synthesized pools of cadherin-7 by treating cells with CHX at a final concentration of 10  $\mu\text{g/ml}$  for 1 h before incubation of the cells with or without competitors for another hour. Under these conditions, we observed a slightly smaller expression of cadherin-7 if the cells were cultured on FN (Fig. 8 B). RGDS peptides (Fig. 8 F) and to a lesser extent anti- $\beta 1$  antibodies (Fig. 8 E) interfered with cell-FN interactions in CHX-treated cells and an increase in cadherin-7 expression at cell-cell contacts was observed. Thus, migrating cad7-29 cells seem to possess a pool of cadherin-7 that can be rapidly mobilized and incorporated into newly formed cell-cell contacts.

For Ncad-1 cells (Fig. 8, bottom panel), anti- $\beta 1$  antibodies caused a decrease in cell spreading which correlated with larger amounts of N-cadherin at cell-cell contacts (Fig. 8 C) than those in untreated cells (Fig. 8 A). A similar result was obtained with RGDS peptides (not shown). Treatment with CHX (Fig. 8 B) gave no significant decrease in the overall level of N-cadherin expressed by the cells. Antibodies against the integrin  $\beta 1$  chain caused an increase in N-cadherin levels at cell-cell contacts (Fig. 8 D). These data suggest that, as for cadherin-7, there may



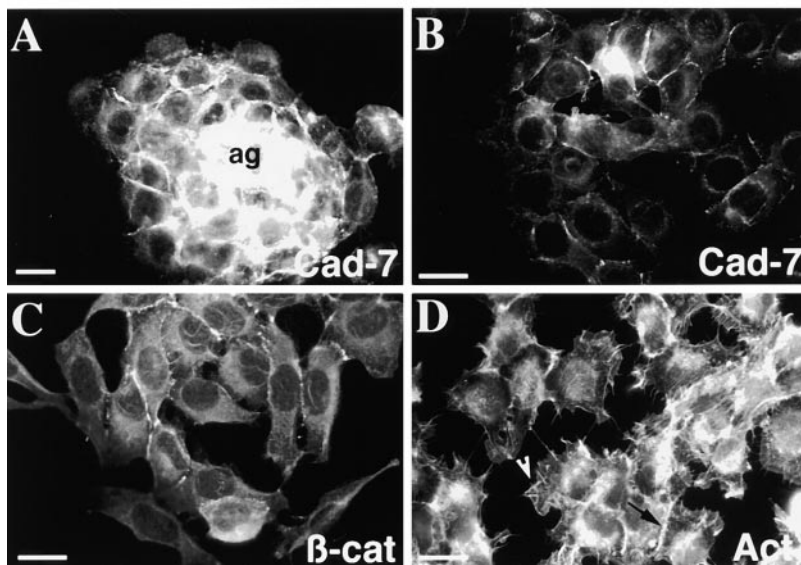
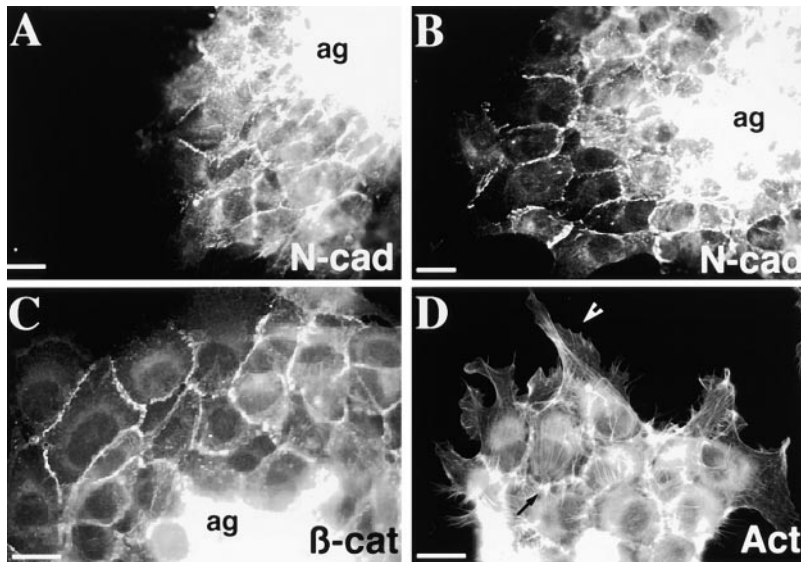
**Figure 6.** Adhesion of cells to FN-coated substrates. (A) Dose-response curve of S180 (black line, white triangles), Ncad-1 (broken line, white circles), and cad7-29 (dotted line, black squares) cells determined for a range of FN concentrations between 0.01 and 0.5  $\mu\text{g/ml}$ . Maximum cell adhesion was achieved at 0.5  $\mu\text{g/ml}$  of FN for the three cell types. Data are expressed as mean percent cell adhesion  $\pm$  the standard error calculated from three independent experiments done in triplicate. (B) Dose-response curve for cell spreading to FN. We measured the extent of cell area in contact with the substrate (arbitrary units) for S180 (white bar), cad7-29 (black bar), and Ncad-1 (gray bar) cells as a function of FN concentration. Data are expressed as mean cellular area  $\pm$  the standard error calculated from at least 30 cells. cad7-29 (D) and Ncad-1 (E) cells were more spread than parental S180 (C) cells on FN (1  $\mu\text{g/ml}$ ).

be a pool of N-cadherin that is rapidly mobilized and incorporated into cell-cell contacts when cell-FN interactions are inhibited. However, N-cadherin levels seem to be less affected than those of cadherin-7 if protein synthesis is inhibited. Western blot analyses of  $\beta$ -catenin, cadherin-7, and N-cadherin levels were carried out with cad7-29 and Ncad-1 extracts. Cell extracts were prepared from untreated cells and from cells treated with 10  $\mu\text{g/ml}$  of CHX for various periods of time (Fig. 9).  $\beta$ -Catenin and cadherin-7 were more rapidly downregulated in cad7-29 cells treated with CHX than in Ncad-1 cells. After 5 h of CHX treatment,  $\beta$ -catenin and cadherin-7 levels in cad7-29 cells had decreased, whereas  $\beta$ -catenin levels were stable and N-cadherin levels were only slightly lower after 5 h in CHX-treated Ncad-1 cells. Thus, the two cadherins seem to have different protein turnover rates and cadherin-7-containing cell-cell contacts have higher turnover rates than N-cadherin-containing cell-cell contacts. This correlated with our videomicroscopy observations that cad7-29 cells established only transient cell-cell contacts, whereas Ncad-1 cells established durable cell-cell contacts. Protein synthesis was required for cad7-29 cells to establish these transients contacts and to maintain the overall level of cadherin-7 during cell migration.

#### ***N-Cadherin-mediated Adhesion Repressed FN-mediated FAK Phosphorylation to a Greater Extent Than Did Cadherin-7***

Isolated cad7-29 and Ncad-1 cells had similar adhesion and spreading properties on FN (see Fig. 6) but had different spreading, scattering, and migratory properties when escaping from aggregates. We analyzed whether N-cadherin- or cadherin-7-mediated cell adhesion differentially affected downstream integrin-signaling molecules. We fo-

cused our study on FAK and its phosphorylation because this protein is one of the principal downstream elements of integrin signaling and is involved in the control of cell adhesion and motility (Guand and Shalloway, 1992; Cary et al., 1996; Gilmore and Romer, 1996; Ilic et al., 1997). We performed Western blots to compare tyrosine-phosphorylated proteins in Ncad-1 and cad7-29 cells engaged in cell-cell and/or cell-FN interactions (Fig. 10). Some proteins in cells plated on FN (lane 2) were specifically tyrosine-phosphorylated, as shown by comparison with cells maintained in suspension (lane 1) or plated on BSA (lane 4). The most highly phosphorylated of these proteins is FAK (arrows, top panels), as previously described by other laboratories. Cells maintained in suspension for 2 h are engaged in strong aggregation process (almost 75%, see also Fig. 2). In this case, one protein with a molecular mass of up to 130 kD appeared to be specifically phosphorylated (Fig. 10, arrowhead). This band was also detected if cells had initiated their cell-cell adhesion process and were plated on BSA, whereas it was not detected in cells plated on FN. Other proteins were found identically phosphorylated in all conditions tested. If Ncad-1 or cad7-29 cells were allowed to initiate cadherin-mediated cell-cell adhesion before plating on FN, there was a decrease in the tyrosine phosphorylation of FAK (lane 3 versus lane 2). Two Western blots were performed and both gave similar results. The blots were stripped and incubated with anti-FAK antibody to determine the amount of FAK in each extract. We then compared, by scanning densitometry, the ratio of phosphorylated FAK to total FAK (Fig. 10, bottom panel) corresponding to the FAK phosphorylation index for untreated cells or cells treated to initiate cell-cell adhesion before plating on FN. N-cadherin-mediated adhesion had a stronger effect than cadherin-7-mediated adhesion on FN-dependent FAK phosphorylation. Thus, the



**Figure 7.** The top panel shows the distribution of N-cadherin (A and B),  $\beta$ -catenin (C), and actin (D) on Ncad-1 cells. The bottom panel shows the distribution of cadherin-7 (A and B),  $\beta$ -catenin (C), and actin (D) on cad7-29 cells. Cell aggregates were cultured on FN (B–D) for 5 h for the two types of aggregates or on uncoated glass coverslips (A) for 5 or 15 h for cad7-29 and Ncad-1 cells, respectively. All Ncad-1 cells produced large amounts of N-cadherin along cell boundaries on both uncoated and FN-coated substrates. F-Actin accumulated in the cortical network along cell-cell contacts, in fibers anchoring at adherens junctions (arrow) and in stress fibers (arrowhead) in lamellipodia. As cad7-29 cells were well dispersed on FN-coated substrate, only the border of the aggregate outgrowth area is shown on B–D (bottom panel). Ag represents the undispersed part of aggregates, which is highly fluorescent under epiillumination. Bars, 20  $\mu$ m.

differences in behavior of aggregated Ncad-1 and cad7-29 cells on FN may be due to different effects of N-cadherin- and cadherin-7-mediated adhesion on integrin-dependent signaling, thereby regulating the initial response of the cells to FN.

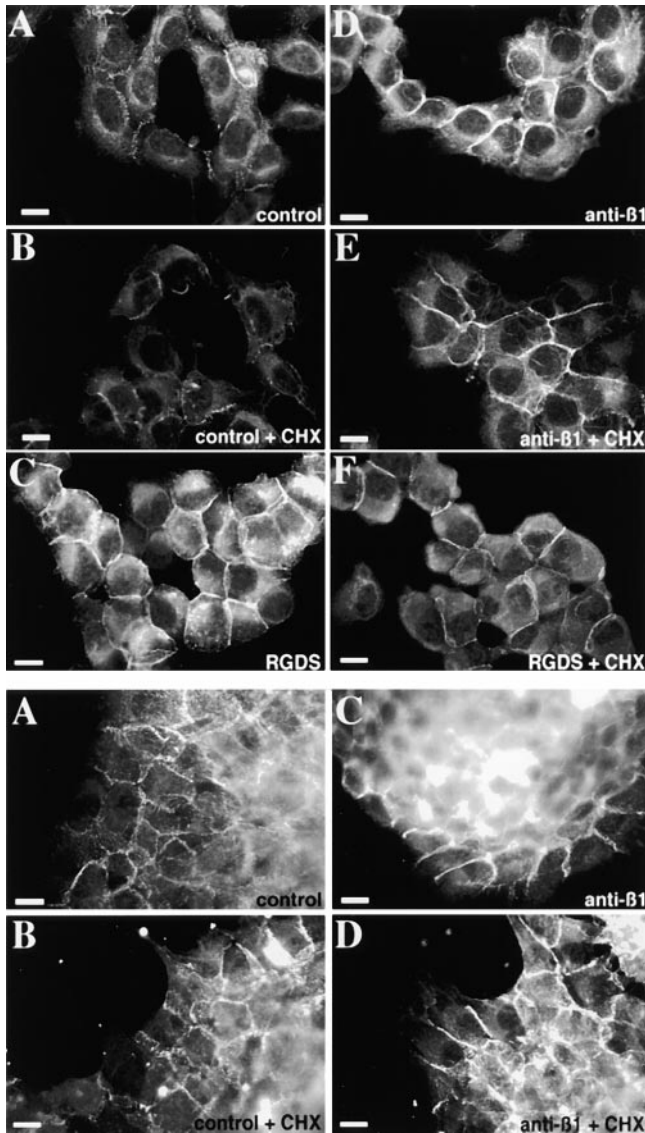
### ***In Vivo Behavior of Transfected Cells***

Small Ncad-1 and cad7-29 aggregates were grafted into embryos to assess their behavior *in vivo*. They were inserted into the NCC migratory pathway, under the ectoderm and between neural tube and somite at the axial level, where NCC were migrating along their ventral pathway (Beauvais et al., 1995). The distribution of cells was established following fixation of the host embryos 18 or 48 h after receiving the graft. For S180 cells, which do not aggregate, we grafted a small piece of a cell pellet obtained by centrifugation.

18 h after the graft, parental S180 cell behavior was as

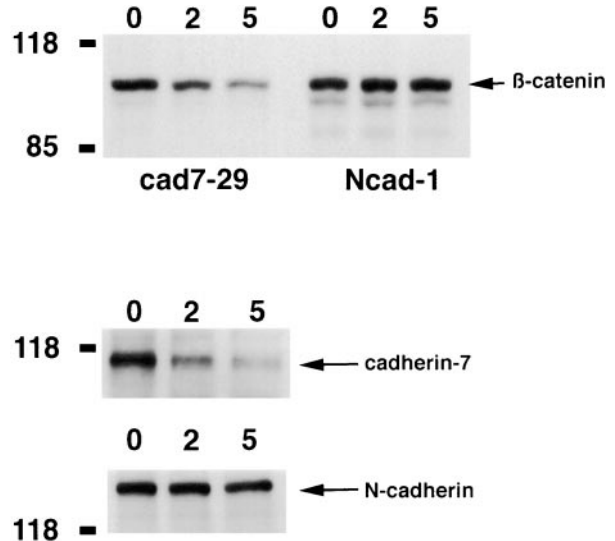
previously described (Beauvais et al., 1995). These cells dispersed from the pellet and migrated as individual cells in the interface between the dermomyotome and sclerotome. They also migrated more ventrally and reached the notochord environment (not shown). After 18 h, cad7-29 cells still formed an aggregate at or close to the graft site (Fig. 11 A, arrowheads). NCC invaded the cad7-29 aggregate, as shown by specific NC-1 immunostaining (Fig. 11, A and B, arrows). This suggests that cad7-29 cells become loosely connected within the aggregate, permitting the penetration of NCC, probably by cadherin-7-mediated interactions. Ncad-1 cell aggregates do not disperse at all into the embryo. They remain as strongly cohesive aggregates at the graft site and NCC do not penetrate the aggregate (not shown). This suggests that cadherin-mediated adhesion functions *in vivo* and that migratory NCC interact transiently with cad7-29, but not with Ncad-1 cells.

Embryos killed 48 h after the graft were used to quantify the aggregate dispersion patterns. We calculated the per-



**Figure 8.** Effect of competitors of cell-FN interactions on the dispersion of cad7-29 (A–F, top panel) and Ncad-1 aggregates (A–D, bottom panel). Cadherin-7 (top panel) and N-cadherin (bottom panel) were detected by CCD7-1 and anti-A-CAM antibody, respectively. Aggregates were allowed to disperse for 5 h on a FN-coated substrate and were then incubated for 1 h with control rat IgG (A and B, both panels), inhibitory rat anti-mouse  $\beta 1$  integrin (D and E, top panel; C and D, bottom panel) each at 100  $\mu\text{g/ml}$  or with RGDS peptides at 2 mg/ml (C and F, top panel). In some cases, cells were incubated for 1 h in the presence of 10  $\mu\text{g/ml}$  CHX before treatment with competitors or control Ig (B, E, and F, top panel; B and D, bottom panel). Bars, 20  $\mu\text{m}$ .

centage of embryos with cells located in arbitrarily defined dorso-ventral regions (Fig. 12, D and E). Cells actively migrating inside embryonic structures (for example in Fig. 12, A and B) were often found scattered within two consecutive levels. In such case, the score in Fig. 12 E corresponds to the maximum level reached by cells. We also investigated whether cad7-29 cells were present at specific sites within the embryo, when compared with parental cells. During these additional 30 h in the embryo, parental



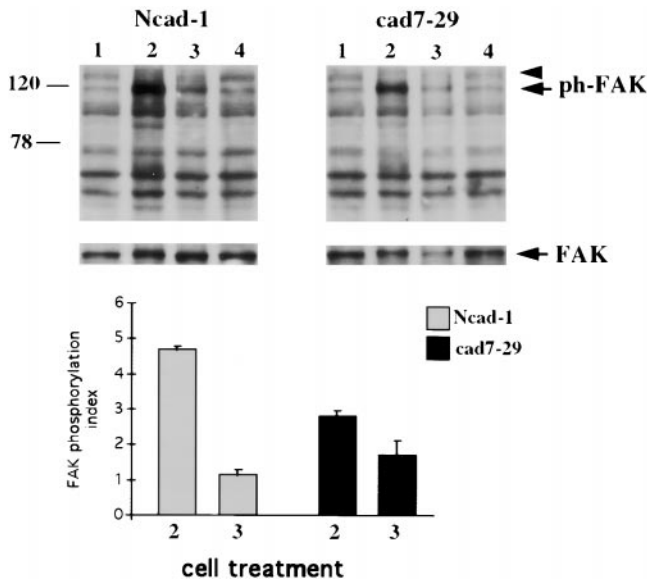
**Figure 9.** cad7-29 cells and Ncad-1 cells were not treated or treated with CHX (10  $\mu\text{g/ml}$ ) for 2–5 h before extraction as described in Materials and Methods.  $\beta$ -Catenin (top), cadherin-7 (middle), and N-cadherin (bottom) levels were determined by Western blot analysis using specific mAbs. Electrophoretic migrations of 118-kD or 85-kD markers are indicated on each panel. Note that over a period of 5 h of CHX treatment, the level of  $\beta$ -catenin and N-cadherin on N-cad1 cells remained similar. In contrast, the levels of both  $\beta$ -catenin and cadherin-7 were down-regulated in treated cad7-29 cells. cad7-29 and Ncad-1 cells produced similar amounts of  $\beta$ -catenin in untreated conditions (0).

S180 cells continued to migrate ventrally. More than 50% of embryos analyzed had cells that had reached levels 3 and 4 and some cells were found closer to the aorta (Fig. 12 A, arrow). cad7-29 cell aggregates were dispersed and cells migrated ventrally, mainly as individual cells (Fig. 12 B, arrow). All embryos analyzed had cells scattered in levels 2 and 3. In almost 90% of embryos injected with Ncad-1 aggregates, there were Ncad-1 cells at or close to the graft site (Fig. 12 C, arrow). Migrating cad7-29 cells in levels 2 and 3 did not incorporate into dorsal root ganglia or associate with dorsal and ventral roots, which produce both N-cadherin and cadherin-7 (Duband et al., 1988; Nakagawa and Takeichi, 1998). Some cad7-29 cells were detected in close contact with ventral roots (Fig. 13, A and B, arrowheads) or dorsal root ganglia but this was also observed for parental S180 cells (Fig. 13, C and D, arrowheads). These results suggest that *in vivo* cad7-29 cells cannot be specifically arrested in cadherin-7-expressing tissue.

## Discussion

N-Cadherin and cadherin-7 are functional in transfected S180 cells. The strong and similar upregulation of  $\beta$ -catenin suggests that Ncad-1 and cad7-29 cells produce similar amounts of cadherins and that their different behaviors *in vitro* and *in vivo* are not primarily due to differences in cadherin synthesis.

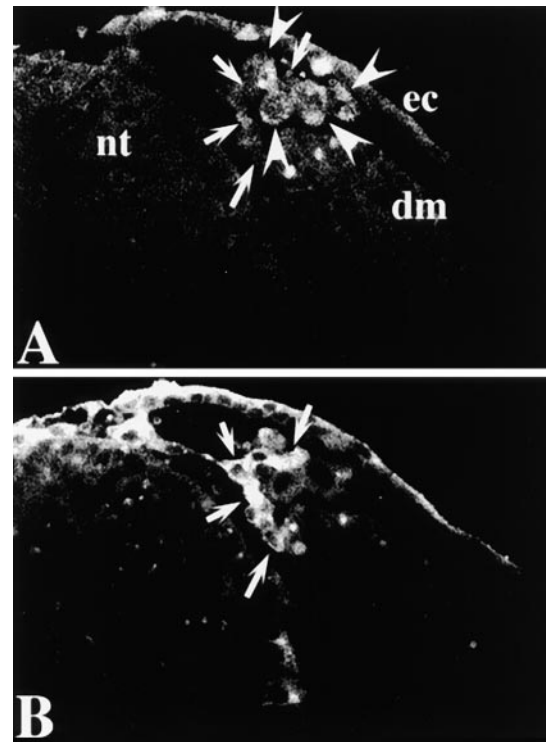
Ncad-1 cells migrated less than cad7-29 cells on FN, which suggests that N-cadherin mediated more stable cell-cell contacts than cadherin-7. This notion was supported



**Figure 10.** Top and middle panels correspond to a Western blot analysis of Ncad-1 and cad7-29 cell extracts with antiphosphotyrosine and anti-FAK antibodies, respectively. Lane 1 corresponds to cells maintained in suspension for 2 h in aggregation conditions. Lane 2 corresponds to isolated cells incubated for 30 min on FN-coated substrate. Lanes 3 and 4 correspond to cells that had only initiated cell-cell adhesion before being deposited for 30 min on FN- or BSA-coated substrate, respectively. The graph shows the FAK phosphorylation index of isolated Ncad-1 (gray bar) and cad7-29 (black bar) cells (2) and cells that had initiated cell-cell adhesion (3) before incubation with FN-coated substrate. The phosphorylation index obtained on BSA serves as a reference. Data are calculated by densitometry of the immunoblots. They are expressed as mean percent index  $\pm$  standard error calculated from two independent blotting analyses.

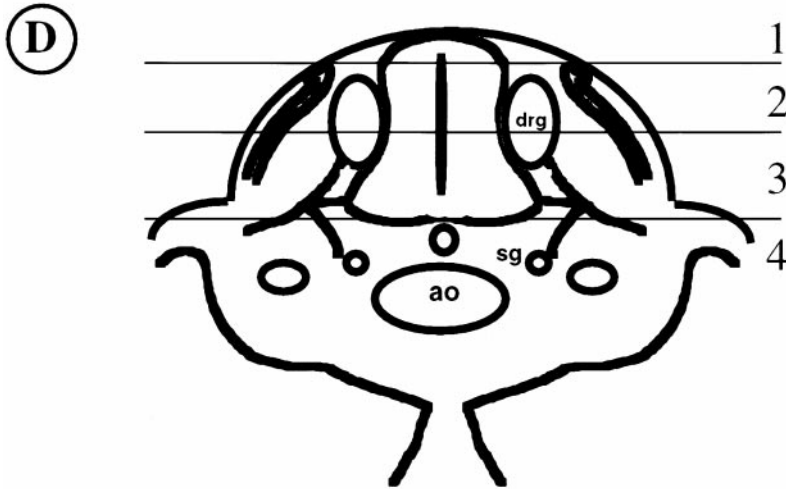
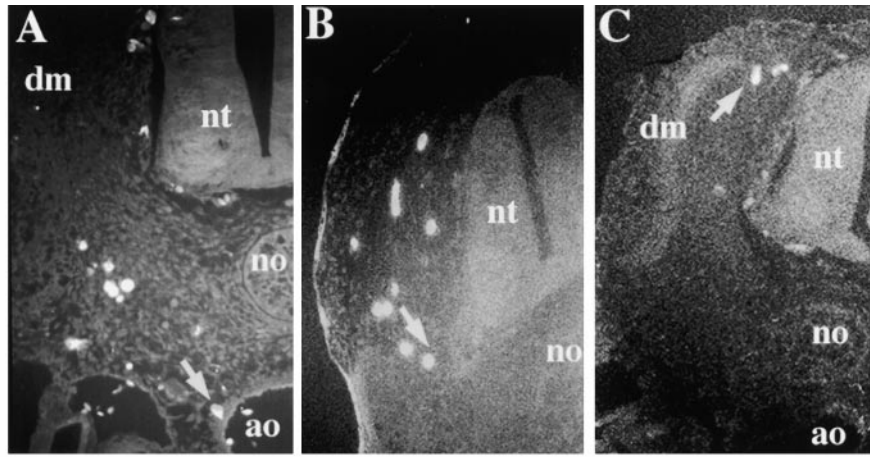
by the observation that the two cadherin types seemed to have different turnover rates, with cadherin-7-based cell-cell contact turnover being higher than that of N-cadherin-mediated cell-cell contacts. Furthermore, cad7-29 migratory cells established transient contacts on FN which may be a major event in the control cell motility by inducing locomotion, as previously suggested (Thomas and Yamada, 1992). Mass cell migrations may require a weak intercellular adhesion system to produce directionality. Differences in the forces generated by cadherin subtype-mediated adhesion may also regulate cell migratory properties. This remains to be demonstrated but our observation that the initial rate of adhesion was lower for cad7-29 than for Ncad-1 cells is consistent with this hypothesis.

N-Cadherin may also directly suppress cell motility, whereas cadherin-7 may not. It has been shown recently for E-cadherin that a portion of the cytoplasmic domain, the juxtamembrane domain, plays a role in the suppression of cell motility (Chen et al., 1997). In addition, if Ncad-1 cells are engaged in cell adhesion before plating on FN, there is a greater reduction in the FN-dependent phosphorylation of FAK than with cad7-29 cells. This is consistent with FAK being involved in the control of cell motility and may account for the fact that Ncad-1 cells escaping from aggregates are less motile and scattered than cad7-29 cells on FN.



**Figure 11.** In vivo behavior of cad7-29 aggregates 18 h after grafting into embryos. Before grafting, cad7-29 cells were labeled with fluorogold and were easily detected in embryonic structures under UV light (A). After fixation, piece of embryos containing the graft were processed for in toto immunofluorescence staining of host NCC with NC-1 antibody (B) and sectioned as described in Materials and Methods. The cells remained as an aggregate at the graft site (A, arrowheads). NCC that had not already migrated more ventrally were found intermixed with cad7-29 cells in the aggregate (A and B, arrows). ec, ectoderm; dm, dermo-myotome; nt, neural tube.

Recently, the molecular basis of cross-talk between integrins and cadherins has been investigated by local stimulation of cell surface cadherins with FN- or cadherin-coated beads. Positive long-range autoregulation of N-cadherin-mediated adhesion has been observed (Levenberg et al., 1998). Conversely, if integrins are stimulated with FN-coated beads, there is a slight reduction in N-cadherin-mediated adhesion in CHO cells (Levenberg et al., 1998). It would be very interesting to test whether this type of FN-dependent signal is more efficient at destabilizing cadherin-7-mediated rather than N-cadherin-mediated adhesions in the cells used here. The perturbation of cad7-29 cell interactions with FN by competitors caused a large increase of cell adhesion and cadherin-7 expression at contact sites. This suggests that the molecular control of cadherin-7-mediated adhesion is also partly dependent upon FN-dependent signaling events, as previously described for N-cadherin expression on migratory NCC (Monier-Gavelle and Duband, 1995, 1997). FN is the more efficient of the diverse ECM components tested for discriminating between Ncad-1 and cad7-29 behavior, which suggests that the molecular control is specific to FN-dependent signaling. The long-range autoregulation observed in CHO cells



**(E)**

cells \ level	number of embryos with cells reaching the levels				number of embryos analyzed
	1	2	3	4	
parental cells S180	0	3	2	2	7
Ncad-1	8	1	0	0	9
Cad7-29	0	4	2	0	6

**Figure 12.** In vivo behavior of parental S180 cells, cad7-29 cells, and Ncad-1 cells, 48 h after grafting into NCC pathways. Fluorogold-labeled cells were detected under UV light. S180 cells migrated ventrally and often reached the aorta (A). cad7-29 cells (B) dispersed and migrated ventrally into the embryonic environment during this additional period of incubation, as shown by comparison with their behavior in embryos after 18 h. In contrast, Ncad-1 aggregates did not disperse and remained close to the graft site (C). (D) Schematic representation of transverse section of an embryo 48 h after grafting and the levels (1–4) used to quantify the maximum ventral migration of grafted cells. (E) Quantification of embryos with cells reaching levels 1–4 by direct determination on embryonic sections. Cells actively migrating inside embryonic structures (for example in A and B) were often found scattered within two consecutive levels. In such cases, level in the table is the maximum level reached by the cells. ao, aorta; drg, dorsal root ganglia; sg, sympathetic ganglia; dm, dermomyotome; no, notochord; nt, neural tube.

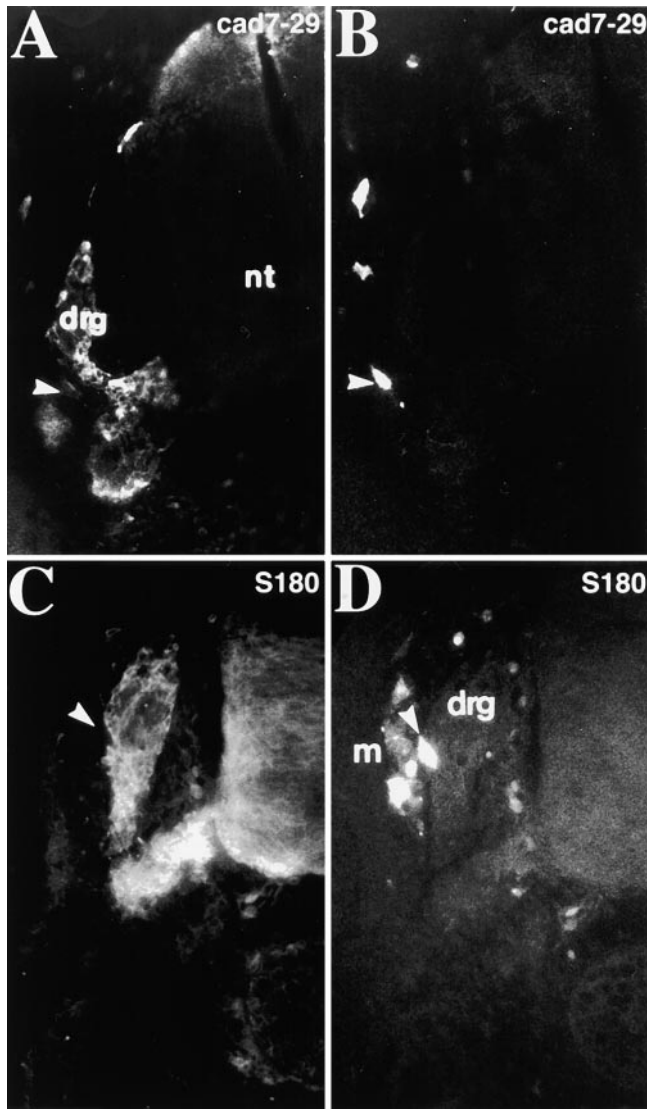
may also occur in Ncad-1 cells to maintain their strong cohesion. It may not occur in cadherin-7-expressing cells but this issue remains to be investigated.

The two types of aggregate also behave differently and in a more drastic manner in vivo than in vitro. Ncad-1 cells did not disperse in vivo. Even after 48 h they remained aggregated at the graft site. 18 h after the graft, parental S180 cells were migrating into embryonic pathways as NCC (although in some aspects they still exhibited distinct features; Beauvais et al., 1995), whereas cad7-29 cells were only found close to the graft site. This result seems to conflict partly with our in vitro data. However, as S180 cells could not self-aggregate, they were injected as a piece of cell pellet, which could not mimic cell aggregate. This

would explain why S180 cells start to migrate as soon as they are injected. No disruption of cell-cell adhesion is required before migration, in contrast to cad7-29 cells. After 18 h, cad7-29 cells seemed to form loosely connected aggregate, suggesting that cell-cell adhesion was being disrupted; mixing was also observed between these cells and NCC, probably mediated by cadherin-7. This phenomenon was not observed for Ncad-1 cells: the aggregate is too cohesive and NCC do not synthesize N-cadherin when they are migrating.

Later, cad7-29 aggregates continued to disperse and the cells started to migrate into embryonic structures. This is consistent with the previous observation that cadherin-7 mRNA is present in migratory NCC and some of their de-





**Figure 13.** Interactions of cad7-29 cells (A and B) and parental S180 cells (C and D) with dorsal root ganglia (drg). Embryos were killed 48 h after the graft, fixed, and labeled with NC-1 to detect drg (A and C). Fluorogold-labeled cells were detected by UV illumination (B and D). There was no specific targeting of cad7-29 cells because both S180 and cad7-29 cells were found in the vicinity of drg (arrowheads). m, myotome; nt, neural tube.

rivatives (Nakagawa and Takeichi, 1995). It has been suggested that cadherin-7 promotes cell sorting in the NCC population and is involved in their targeting. In this study, we observed that cad7-29 cells interacted transiently with endogenous NCC before migrating ventrally into embryonic structures but were not specifically arrested to the vicinity of ventral roots or dorsal root ganglia (structures expressing cadherin-7). Thus, cadherin-7 may be more crucial for the migration than for NCC targeting to specific sites, especially if the ECM environment contains a large amount of FN. It has been shown that FN and type I collagen are particularly abundant along the migratory pathways of NCC, whereas laminin and type IV collagen are found in the basal surfaces of epithelia but are rarely associated with NCC. The different effects of FN and type I

collagen on in vitro cad7-29 cell aggregate dispersion suggest that FN rather than type I collagen is the principal mediator of cad7-29 cell motility in vivo.

Large amounts of cadherin-7 are expressed in dorsal root ganglia raising the question of its role in tissue formation. Cadherin-7 may well have a role in the maintenance of cell-cell adhesion between neuronal cells because FN levels are very low in the ganglia (Thiery et al., 1982). However, N-cadherin is more likely than cadherin-7 to be involved in the aggregation of NCC. Ncad-1 cells cannot disperse in NCC pathways. This inhibition of cell migration in cells that express N-cadherin contrasts with the ability of N-cadherin substrates to promote neuritic outgrowth, although neurite elongation occurs by a different mechanism: new membranes are added at the base of the growth cone with the neurite shaft remaining immobile (Doherty and Walsh, 1991). However, it correlates with the fact that NCC cease to produce N-cadherin before they emigrate from neural tube. In a recent study, Nakagawa and Takeichi (1998) observed ectopic expression of N-cadherin on isolated migratory NCC in the ventral pathway after injecting recombinant adenovirus into the lumen of the neural tube. Nevertheless, as the authors themselves pointed out, the lag time between adenovirus infection and protein synthesis may have induced N-cadherin synthesis at a late stage of NCC migration.

Our observations that cadherin-7-mediated cell adhesion is regulated by the ECM environment and that N-cadherin and cadherin-7 adhesion differentially affect FN-dependent signaling indicate another way of regulating cell behavior in vivo. It would be very interesting to investigate the role of other cadherins such as cadherin-11, produced in large amounts by mesenchymal and migratory cells in vitro and in vivo, and to analyze whether behavior on ECM components differs. The analysis of molecules differentially involved in the cross-talk between type I and type II cadherin-mediated adhesion and integrin signaling should provide insight into the molecular mechanisms controlling tissue remodeling and cell migration during early embryogenesis.

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