Journal of Cellular Biochemistry 78:566–577 (2000)

Relative Abundance of Different Cadherins Defines Differentiation of Mesenchymal Precursors Into Osteogenic, Myogenic, or Adipogenic Pathways

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Abstract Cadherins, a family of cell-cell adhesion molecules, provide recognition signals that are important for cell sorting and aggregation during tissue development. This study was performed to determine whether distinct cadherin repertoires define tissue-specific lineages during differentiation of immature C3H10T1/2 and C2C12 mesenchymal cells. Both cell lines expressed mRNA for N-cadherin (N-cad), cadherin-11 (C11), and R-cadherin (R-cad). After induction of osteogenesis by recombinant human BMP-2 (rhBMP-2) treatment, steady state N-cad mRNA slightly increased in C3H10T1/2 cells. Likewise, the abundance of C11 mRNA increased in both cell lines, although the changes were more remarkable in C2C12 cells. By contrast, R-cad expression was almost shut off by rhBMP-2. The immature but committed osteoblastic MC3T3-E1 cells exhibited only minor changes in N-cad and C11 mRNA abundance after rhBMP-2 treatment. Whereas adipogenic differentiation was associated with a net decrease of N-cad and C11 expression in C3H10T1/2 cells, induction of myogenesis in C2C12 cells resulted in up-regulation of N-cad, while R-cad mRNA became undetectable in either case. Similarly, the adipocytic 3T3-L1 cells expressed very low levels of all cadherins when fully differentiated. Therefore, the repertoire of cadherins present in undifferentiated mesenchymal cells undergoes distinct changes during transition to mature cell phenotypes. Although neither N-cad nor C11 represent strict tissue-specific markers, the relative abundance of these mesenchymal cadherins defines lineage-specific signatures, perhaps providing recognition signals for aggregation and differentiation of committed precursors. J. Cell. Biochem. 78:566–577, 2000.

Key words: osteoblast; adipocytes; myoblasts; cell-cell adhesion; cadherin

INTRODUCTION

Cell-cell and cell-matrix interactions are key events in embryogenesis and tissue differentiation and are mediated by an array of cell adhesion molecules. Cadherins are integral membrane proteins with a large extracellular domain that allows calcium-dependent homophilic cell-cell adhesion, a single transmembrane domain, and a short intracellular tail linked to the actin cytoskeleton via interactions with other proteins, catenins [Gumbiner, 1988; Takeichi, 1994; Ellerington et al., 1996]. Expression of certain cadherins during embryogenesis and tissue differentiation provides specific recognition signals that allow cells committed to a certain lineage to sort out and aggregate at specific sites [Gumbiner et al., 1988; Takeichi, 1994; Ellerington et al., 1996]. To date, more than a dozen cadherins have been identified, all of which are the products of different genes. Based on structural differences in their extracellular domains, cadherins are now classified into type I and type II, although it is still uncertain whether such classification reflects functional differences [Ellerington et al., 1996].

We have previously reported that human osteoblasts and bone marrow stromal cells ex-

Part of this work has been presented in abstract form at the 21st annual meeting of the American Society for Bone and Mineral Research, St. Louis, Missouri, September 30 - October 4, 1999; Abstract #SU187.

Grant sponsor: National Institutes of Health; Grant number: AR43470; Grant number: AR32087.

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Received 30 December 1999; Accepted 29 February 2000

TABLE I PCR Primer Sequences used for cDNA Probe Preparation*

		Primer Sequence	Product size (bp)
N-cad	Forward (1329–1356)	5′-GTGGCAGCTGGTCTGGATCGAGAGAAAG-3′	552
	Reverse (1854–1881)	5′-TCAGCATGGTACCTGCGTGGAGGCCTTC-3′	
C11	Forward (862–889)	5′-AGCGGTACCACTGGAGCCACCTTCAGAA-3′	540
	Reverse (1375–1392)	5′-CATGACGCGTTCTATGCCGTCTCCATCA-3′	
R-cad	Forward (1838–1863)	5′-CTGGGACCCTGCAGATCTACCTCATT-3′	884
	Reverse (2697–2722)	5′-AATCTTGATCCCCGGAGCTGGAGGAG-3′	
Adipsin	Forward (1587–1604)	5′-CATGGGTACCTATCCCAGAATGCCTCGT-3′	274
	Reverse (1824–1841)	5′-CATGACGCGTGCAAGTGTCCCTGCGGTT-3′	
MCK	Forward (741–768)	5′-AAGCTTCCTTGTGTGGGTGAACGAGGAG-3′	390
	Reverse (1104–1131)	5′-CACCAGCTGCACCTGTTCGACTTCGGAT-3′	

*All sequences are from mouse genes.

press a repertoire of cadherins, including N-cad, C11, and cadherin-4 (C4), and that the expression pattern of osteoblast cadherins changes during differentiation in vitro [Cheng et al., 1998]. Using inhibitory peptides and a dominant negative N-cadherin construct, we have demonstrated that interference with cadherin-dependent cell-cell adhesion results in altered bone matrix protein expression and decreased matrix mineralization in the mouse osteoblastic MC3T3-E1 cells [Cheng et al., 1998] [Cheng et al., submitted]. Since MC3T3-E1 cells represent immature but committed osteoblastic cells, these results suggest that cadherins are required for progression of the osteoblastic differentiation program to its full maturity. However, the question remains as to whether cadherins are also physiologically involved in determining the initial fate of uncommitted precursors to the different lineages originating from mesenchymal progenitors. In this context, the biologic diversity of cadherins expressed by osteoblasts may provide a distinct repertoire of adhesion molecules allowing committed yet undifferentiated osteogenic precursors to sort out from other cells and aggregate. In adult tissue, this multimolecular signature could direct osteogenic precursors towards areas of the bone surface undergoing new bone formation.

Osteoblasts are derived from common mesenchymal progenitors that can also differentiate into chondroblasts, myoblasts, and adipocytes [Grigoriadis et al., 1988; Grigoriadis et al., 1990; Yamaguchi and Kahn, 1991]. Both N-cad and C11 are expressed during embryonic development in mesenchymal cells and are believed to participate in chondro-osteogenic cell condensation in somites and limb buds [Oberlender and Tuan, 1994a]. The distribution of these two cadherins largely overlaps during embryogenesis, although C11 is particularly abundant in areas undergoing chondrogenesis [Oberlender and Tuan, 1994a]. In this study, we used both the C3H10T1/2 cell line, which can be induced to differentiate towards either osteogenic or adipogenic pathways, and C2C12 cells, representing immature myoblasts with chondro-osteogenic potential, as models of pluripotent mesenchymal precursors. We found that mesenchymal progenitor cells express N-cad, C11, and R-cad, the mouse homologue of human C4. Although neither N-cad nor C11 represents strict tissue-specific markers, the relative abundance of these mesenchymal cadherins changes depending on commitment to the different lineages. The results are consistent with the hypothesis that different cadherin repertoires provide specific recognition signals for cell aggregation and differentiation among tissues of mesenchymal origin.

MATERIALS AND METHODS

Reagents and Chemicals

Complementary DNA probes for mouse N-cad, C11, R-cad, muscle creatinine kinase (MCK), and adipsin were prepared by reverse transcription and PCR from mouse brain (N-cad), calvaria (C11), eye (R-cad), muscle (MCK), and fat tissue (adipsin). Oligonucleotide primers used for PCR are listed in Table 1. After tissue homogenization, total RNA was isolated using the TRI reagent (Molecular Research Center, Cincinnati, OH), and first-strand cDNA was synthesized by the Reverse

Transcription System kit (Promega, Madison, WI) according to manufacturer's instructions. PCR consisted of 30 cycles with settings of 94°C for 30 s, 55°C for 45 s, and 72°C for one min. The cDNA probe for mouse osteocalcin (OC) was kindly provided by Dr. John Wozney (Genetics Institute, Cambridge, MA) and the cDNA for mouse β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Clontech Laboratories (Palo Alto, CA). The mouse monoclonal anti-pan cadherin antibody (C1821) raised against the 24 amino acids at the C-terminus of chicken N-cad was purchased from Sigma Chemical (St. Louis, MO). A goat polyclonal antibody against C11 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human bone morphogenetic protein 2 (rhBMP-2) was kindly provided by Dr. Vicki Rosen, of the Genetics Institute. Unless otherwise indicated, all the other chemicals, including the tissue culture medium, were from Sigma Chemical (St. Louis, MO).

Induction of Chondro-osteogenic and Adipogenic Differentiation in C3H10T1/2 Cells

The murine embryonic mesenchymal cells C3H10T1/2 (American Type Culture Collection, Manassas, VA), are pluripotent cells that retain an immature, fibroblast-like appearance under standard tissue culture conditions but can differentiate into osteoblast, chondrocyte, adipocyte, and myoblast with specific inducers [Ahrens et al., 1993; Katagiri et al., 1990; Taylor and Jones, 1979]. For these studies, C3H10T1/2 cells were grown in Basal Medium of Eagle (BME) (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO) and antibiotics (100 U/ml of penicillin-G and 100μg/ml of streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded at 2 x 10^4 cells per cm^2. For osteochondrogenic differentiation, the culture medium was changed to a fresh medium containing 100ng/ml rhBMP-2 16 h after plating. Adipogenic differentiation was initiated 24–48 h after confluence by changing to a BME medium containing 10% FBS, 5 μg/ml insulin, 50 μM indomethacin, and 0.1 μM dexamethasone for at least nine days.

Induction of Osteochondrogenic and Myogenic Differentiation in C2C12 Cells

The mouse myoblast cell line, C2C12 (American Type Culture Collection, Manassas, VA) represents committed cells that differentiate rapidly into myoblasts after reaching confluence when grown in media containing low (5%) serum concentration [Katagiri et al., 1994]. However, treatment with rhBMP-2 can redirect their differentiation pathway towards osteoblasts [Katagiri et al., 1994]. Accordingly, C2C12 cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 15% FBS (growth medium) and antibiotics. The cells were seeded at 2 x 10^4 cells per cm^2 and cultured in growth medium. For osteochondrogenic differentiation, the culture medium was changed to a fresh medium containing 5% FBS (low mitogenic medium) with rhBMP-2 (300ng/ml) 16 h after plating. Conversely, growth medium was switched to low mitogenic medium without rhBMP-2 for myogenic differentiation.

Osteoblastic and Preadipocytic Cell Lines

The mouse MC3T3-E1 osteoblastic cells were derived from spontaneous immortalization of calvaria cells selected by the 3T3 passaging protocol, and represent immature osteogenic cells that can differentiate into mature osteoblast [Sudo et al., 1983]. MC3T3-E1 cells were maintained in DMEM/F12 medium with 10% FBS either with or without rhBMP-2 treatment. To stimulate osteoblast differentiation, cells were seeded in p100 dished (3 x 10^6 cells/dish) and 16 h after plating the medium was changed to fresh medium containing 100 ng/ml rhBMP-2. The 3T3-L1 preadipocytic cell line was a kind gift of Dr. Clay Semenkovich (Washington University, St. Louis, MO). These cells were maintained in an immature state by culturing in DMEM supplemented with 20% FBS, 2.0 mM glutamine, and antibiotics. Differentiation was initiated 24 and 48 hrs after confluence by changing the medium to DMDM containing 10% FBS, 5 μg/ml insulin, 50 μM indomethacin, and 0.1 μM dexamethasone [Tordjman et al., 1989]. This medium was changed to growth medium (DMEM supplemented with 10% FBS) 48 hrs later, and the growth medium was replaced every two days thereafter. The medium containing inducers was applied only for 48 hrs. And then, replaced

with medium without inducers. Cells were harvested five and 10 days after treatment.

Histochemical Analysis

For alkaline phosphatase activity (chondro-osteogenic marker), cells were fixed for 30 s in citrate-buffered acetone (60%) at room temperature. After washing in deionized water, cells were incubated for 30 min with a mixture of 0.6 mg/ml of fast blue RR salt and 0.1 mg/ml of naphthol AS-MX phosphate (Sigma, St. Louis, MO) in water at room temperature. For Oil Red O staining (adipogenic marker), C3H10T1/2 cells were fixed with 10% buffered neutral formalin for 5 min at room temperature and rinsed in distilled water. Working stock solution of Oil Red O was prepared from 1% (wt/vol) Oil Red O in 99% isopropanol and diluted to 0.3% (vol/vol) with distilled water. Cultures were stained with Oil Red O working solution for 15 min with gentle rocking and rinsed with distilled water. For May Grunwald-Giemsa staining, cells were fixed with methanol for 15 min and stained with May Grunwald stain solution (Sigma, St. Louis, MO) for 5 min. After washing with phosphate buffer saline (PBS) for 3 min, cells were placed in 1:20 diluted Giemsa Stain solution (Sigma, St. Louis, MO), rinsed, and air-dried.

Alkaline Phosphatase Activity

Confluent cells in 24 well plates were washed three times with Tris buffer saline (TBS) (50 mM Tris, pH 7.4, and 0.15 M NaCl) and stored at –20 °C until assayed. As previously described [Cheng et al., 1994a], the cell layer from each well was scraped into 0.5 ml of 10 mM Tris, pH 7.4, containing 0.5 mM $MgCl_2$ and 0.1% TritonX-100 and sonicated with a Fisher Dismembranetor (at 30-40% of maximum strength). Alkaline phosphatase activity was measured using p-nitrophenyl phosphate (3 mM) as substrate in 0.7 M 2-amino-2-methyl-1-propanol, pH 10.3, and 6.7 mM $MgCl_2$, and expressed as p-nitrophenol produced in nmol/min/mg protein. Protein was measured using the Bio-Rad method (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

Northern Analysis

Total RNA was isolated from cultured cells using the TRI reagent (Molecular Research Center, Cincinnati, OH) and separated by agarose gel electrophoresis ([Cheng et al., 1994b] 20 μg/lane). Northern blot analysis was performed as described previously [Cheng et al., 1998] using the cDNA probes detailed above. Autoradiographs were digitized and the intensity of reactive bands was quantified by densitometric analysis after normalization for either mouse β-actin or GAPDH using SigmaGel software (Jandel Scientific, Chicago, IL). Results are representative of three independent experiments.

Western Analysis

A procedure previously described was used [Cheng et al., 1998]. Briefly, cells grown on 100 cm^2 petri dishes were extracted in a buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 1 % Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Proteins were separated by 10 % SDS-PAGE, and electroblotted to a nitrocellulose membrane. Membranes were blocked with 0.1% Tween-20 TBS containing 2% BSA and 3% dry milk, pH 7.4, for one h. Either a mouse monoclonal antibody against chicken N-cad (1:200), or a goat polyclonal antibody against C11 in blocking buffer (1:200) was added and the incubation continued for another hour. After washing in 0.1% Tween-20 TBS, membranes were incubated with horseradish peroxidase-conjugated antimouse or antigoat antibodies (1:2000) for one h. After extensive washing, bands were visualized by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech, NJ).

RESULTS

Induction of Differentiation in Immature C3H10T1/2 and C2C12 Cells

Whereas C3H10T1/2 cells maintained on regular medium did not change their fibroblastic morphology and grew in a monolayer (Fig. 1A and B), BMP-2 treated cells lost their fibroblastic features, assuming a rounded appearance and forming multiple cell layers in some areas (Fig. 1C). These morphologic changes appeared 24 h after confluence and became more evident with time. Seven days postconfluence, alkaline phosphatase activity was detected in more than 15% of the cell population by cytochemical staining (Fig. 1E), whereas no Alcian blue stain, specific for cartilage matrix, was observed (not shown). Alkaline phosphatase

activity in cell lysates was 104±35 nmol of p-NP/min/mg protein (n=3) in rhBMP-2 treated cells, whereas confluent cells grown in the absence of rhBMP-2 showed no detectable activity.

C3H10T1/2 cells treated with dexamethasone, indomethacin, and insulin underwent adipocytic differentiation as shown by the presence of cytoplasmic lipid droplets evident in phase-contrast micrographs (Fig. 1D) and after Oil Red O staining (Fig. 1F). Foci of adipocytes began appearing three days after treatment and both adipocyte number and lipid droplet size increased with time in culture. By day nine, more than 80% of the cells on a dish contained Oil Red O positive lipid droplets. Likewise, treatment of 3T3-L1 cells with insulin and dexamethasone resulted in adipocyte conversion of >90% of the cell population by day 10, as assessed by morphologic examination and Oil Red O stain (not shown).

Similar to C3H10T1/2 cells, immature C2C12 cells also appeared fibroblast-like in the absence of inducers (Fig. 2A and B). Myogenic differentiation was induced by culturing C2C12 cells in low mitogenic medium (5% FBS), as demonstrated by the appearance of numerous multinucleated myotubes starting at day four after reduction of serum concentration in the medium. An extensive network of tubular structures was formed in cells cultured up to four weeks (Fig. 2D and E). Under such culture conditions, these cells were negative for alkaline phosphatase (not shown). On the other hand, cells grown in the presence of rhBMP-2 (300 ng/ml) did not form any myotubes, growing instead in multilayers similar to what we observed with rhBMP-2 treated C3H10T1/2 cells (Fig. 2C and 1C). In these conditions, more than 85% of cells were alkaline phosphatase positive by cytochemical staining (Fig. 2E), with an average enzymatic activity of 527 ±114 nmol of p-NP/min/mg protein (n =3) compared to undetectable levels in control cells. On the contrary, the immature osteoblastic MC3T3-E1 cells did not show remarkable phenotypic difference after treatment with rhBMP-2 (100 ng/ml) for seven days (not shown).

Analysis of Cadherin Expression in Differentiating C3H10T1/2 and C2C12 Cells

To investigate the pattern of cadherin expression during osteogenic and adipogenic differentiation, we monitored the accumulation of mRNA, N-cad, C11, and R-cad, as well as markers specific for osteoblasts (osteocalcin) and adipocytes (adipsin). As shown in Fig. 3, undifferentiated, proliferative C3H10T1/2 cells expressed mRNA for all three cadherins, and steady state levels of both N-cad and C11 mRNA were slightly increased by rhBMP-2 treatment. By contrast, R-cad expression, which was very low during the proliferative stage, was almost shut off by rhBMP-2 treatment. These changes were associated with the induction of osteocalcin mRNA expression and alkaline phosphatase activity (Fig 1E). Likewise, both N-cad and C11 were constitutively expressed in confluent, mature, mineralizing MC3T3-E1 cells and the abundance of both cadherins was not significantly altered by treatment with rhBMP-2, whereas osteocalcin expression was massively increased. Induction of adipogenic differentiation (demonstrated by expression of adipsin mRNA) in C3H10T1/2 cells was associated with significant down-regulation of all three cadherins, although substantial amounts of C11 mRNA were still present in these conditions. Similar changes occurred in differentiating 3T3-L1 cells, and expression of all three cadherins, including C11, was significantly down-regulated by day 10, in conjunction with robust induction of adipsin (Fig. 3).

Undifferentiated, proliferative C2C12 cells also expressed mRNA for N-cad, C11, and R-cad (Fig. 4). Aside from a slight increase of C11 mRNA, the abundance of N-cad and R-cad did not change after the cells reached confluence. Such findings are quite similar to those just described for C3H10T1/2. In cells cultured in low mitogenic medium for seven days, the abundance of N-cad was increased, whereas the C11 expression did not increase until three weeks later. R-cad mRNA became almost undetectable. MCK mRNA, a specific marker for myogenic development, was detected at one week and increased at two weeks without further changes thereafter. After treatment with rhBMP-2, N-cad mRNA was down-regulated, whereas expression of C11 mRNA increased. By contrast, R-cad expression was almost shut off by rhBMP-2. These changes were associated with robust induction of osteocalcin mRNA (Fig. 4) and alkaline phosphatase activity (not shown). The relative expression of N-cad and C11 in rhBMP-2-treated C2C12 cells was

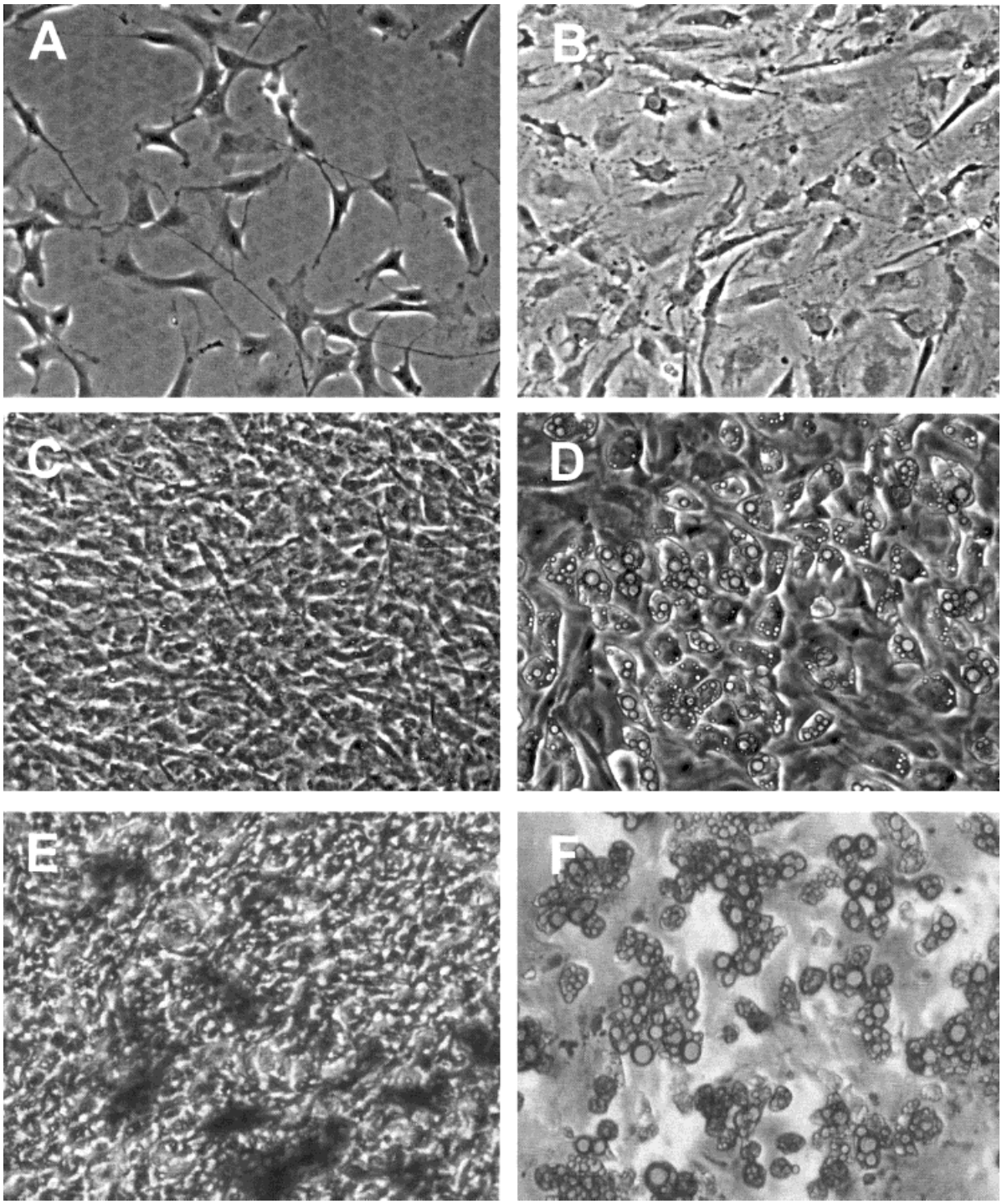

Fig. 1. Histochemical changes in differentiating C3H10T1/2 cells. Phase contrast micrograph of C3H10T1/2 cell in the proliferative (**A**) and confluent (**B**) stages maintained in growth medium. Phase contrast (**C**) and alkaline phosphatase stain (**E**) in confluent cells after treatment with rhBMP-2 (100 ng/ml) for seven days. Alkaline phosphatase activity is represented by the dark cytoplasmic stain. Phase contrast (**D**) and Oil Red O stain (**F**) in confluent cells after treatment with 0.1 μM dexamethasone, 50 μM indomethacin, and five μg/ml insulin for nine days. Adipocytes are recognizable by the presence of gray, cytoplasmic lipid droplets. Objective magnification X100.

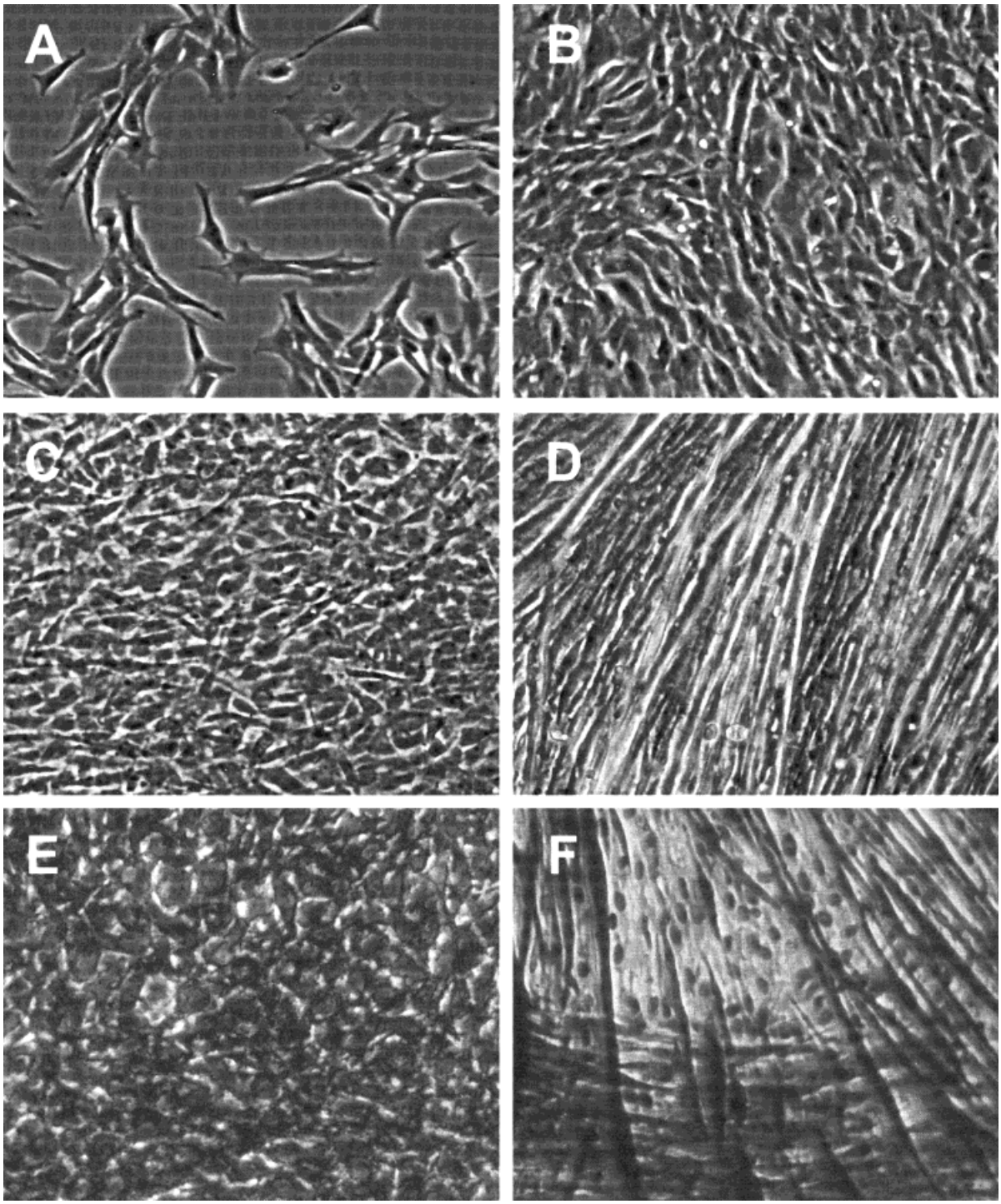

Fig. 2. Histochemical changes in differentiating C2C12 cells. Phase contrast micrograph of C2C12 cells in proliferative (**A**) and confluent (**B**) stages. Note the morphologic similarity with proliferative C3H10T1/2 cells in Fig. 1. Phase contrast (**C**) and alkaline phosphatase stain (**E**) after treatment with rhBMP-2 (300 ng/ml). Most (>80%) cells in this confluent culture are positive for alkaline phosphatase. Phase contrast (**D**) and May-Grunwald-Giemsa stain (**F**) after incubation for 14 days in low mitogenic medium (containing 5% FBS) showing numerous multinucleated myotubes (dark stain in F). Objective magnification X100.

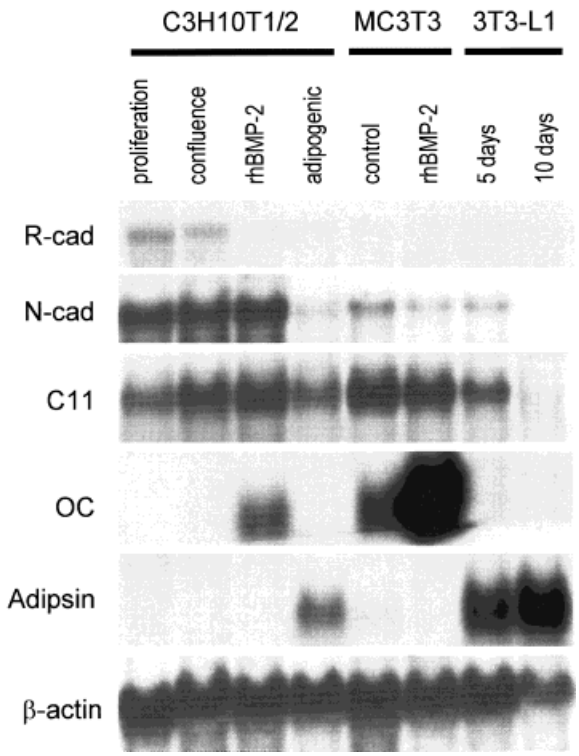

Fig. 3. Expression of cadherins and tissue specific marker gene products in C3H10T1/2 cells during chondro-osteogenic and adipogenic differentiation. Total RNA was purified from proliferative and confluent undifferentiated C3H10T1/2 cells, as well as from cultures treated with either rhBMP-2 (100 ng/ml) or an adipogenic cocktail (indomethacin, dexamethasone, and insulin) for the time indicated and separated on agarose gels. Total RNA was also isolated from MC3T3 osteoblastic cells grown in the presence or in the absence of rhBMP-2 (100 ng/ml), and from 3T3-L1 adipocytic cells, five and 10 days after induction of differentiation by insulin and dexamethasone. After transfer onto nylon membranes, mRNA was hybridized with ^{32}P-labeled cDNA probes for R-cad, N-cad, C11, OC, adipsin, and β-actin. After each hybridization step, the membranes were extensively washed and re-exposed to X-rays to ensure complete removal of the previous hybridization band. The results are representative of three independent experiments.

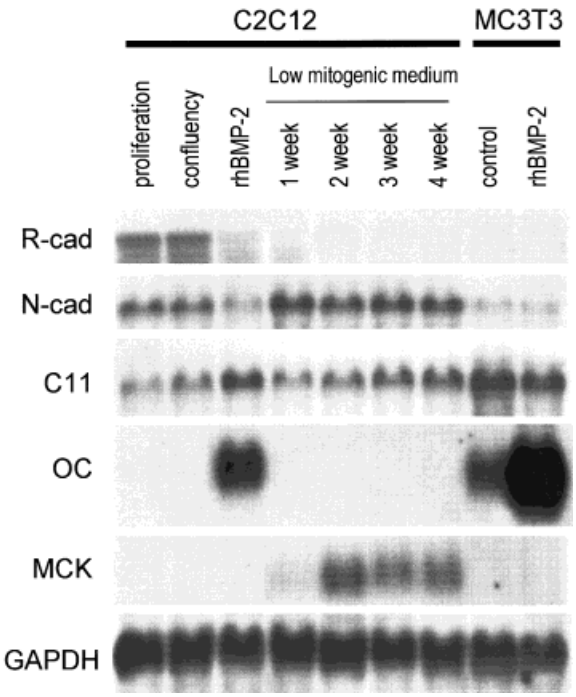

Fig. 4. Expression of cadherins and tissue specific markers in C2C12 cells during chondro-osteogenic and myogenic differentiation. Total RNA was purified from proliferative and confluent undifferentiated C3H10T1/2 cells, as well as from cultures grown in the presence of either rhBMP-2 (300 ng/ml) or low mitogenic medium for the time indicated and then separated on agarose gels. Total RNA was also isolated from MC3T3 osteoblastic cells grown in the presence or in the absence of rhBMP-2 (100 ng/ml). After transfer onto nylon membranes, mRNA was hybridized with ^{32}P-labeled cDNA probe for R-cad, N-cad, C11, OC, MCK, and GAPDH. After each hybridization step, the membranes were extensively washed and re-exposed to X-rays to ensure complete removal of the previous hybridization band. The results are representative of three independent experiments.

closely similar to that observed in MC3T3-E1 cells, although C11 mRNA abundance was slightly higher in unstimulated MC3T3-E1 than in stimulated C2C12 cells. As expected, exposure to rhBMP-2 produced massive induction of osteocalcin expression, while C11 abundance only slightly decreased.

A pan-cadherin antibody, raised against a synthetic peptide corresponding to the C-terminal amino acids of chicken N-cadherin reacted with a distinct single band at around 135 kD in both C3H10T1/2 and C2C12 cell lysates (Fig. 5A). The intensity of this band increased in rhBMP-2-treated C3H10T1/2 cells and in myogenic C2C12 cells, but decreased in adipogenic C3H10T1/2 cells. The molecular size of this band, as well as an expression pattern consistent with that of N-cad mRNA, strongly suggest that this band corresponds to N-cad. However, since this antibody is not exclusively specific for N-cad, we cannot exclude that other cadherins may be present in the reactive band. As shown in Fig. 5B, expression of C11 protein was stimulated by treatment with rhBMP-2 in both C3H10T1/2 and C2C12 cells, whereas it was decreased in C3H10T1/2 cells undergoing adipogenic differentiation and did not change in myogenic C2C12 cells at all. These results are in full agreement with the changes observed in mRNA levels.

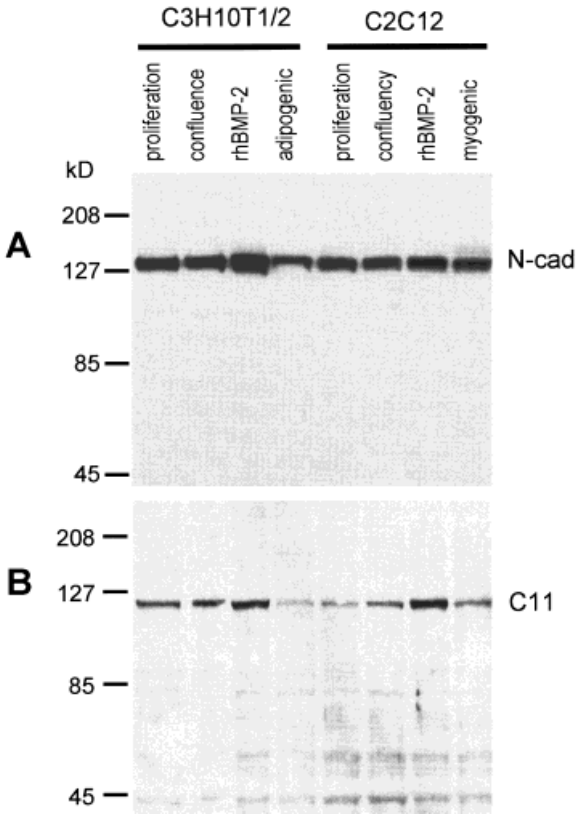

Fig. 5. Expression of N-cad and C11 proteins in C3H10T1/2 and C2C12 cells induced towards three differentiation pathways. Treatments with rhBMP-2, adipogenic, or myogenic media were performed as detailed in the text and summarized in the previous figures. Whole cell lysates were separated by SDS-PAGE and blotted with either a monoclonal anti-pan cadherin antibody, which recognizes the C-terminal tail of chicken N-cadherin (**A**), or a goat polyclonal anti-mouse C11 antibody (**B**). The results are representative of three independent experiments.

DISCUSSION

In this study, we have demonstrated that undifferentiated mouse mesenchymal cells express various cadherins and that the relative expression of these molecules changes during osteogenic, adipogenic, or myogenic differentiation. In particular, we observed increased expression of C11 and N-cad during osteogenic and myogenic differentiation, but consistent down-regulation of all cadherins during adipogenesis. Relative to N-cad, the abundance of C11 was higher in cells committing to the chondro-osteogenic pathway, whereas the reverse was observed in myogenic cells. R-cad expression was strongly inhibited in all differentiation pathways.

The presence of multiple cadherins in immature mesenchymal cells is consistent with observations in embryo sections demonstrating abundant expression of N-cad and C11 in areas undergoing myogenic and chondo-osteogenic differentiation. In particular, chondrogenesis has been associated with a five-fold increase in N-cad expression during the period of cellular condensation in limb buds [Oberlender and Tuan, 1994a], and a similar increase in N-cad expression occurs during in vitro chondrogenesis stimulated by BMP-2 in C3H10T1/2 cells [Haas and Tuan, 1999]. In our studies, rhBMP-2 treatment resulted in increased N-cad expression in C3H10T1/2 cells, but slightly decreased N-cad abundance in C2C12 cells. Although N-cad expression is increased at an early stage of chondrogenic condensation during limb bud development, its level is down-regulated in later stages [Oberlender and Tuan, 1994b; Oberlender and Tuan, 1994a; Tsonis et al., 1994]. Accordingly, the increased expression of N-cad in rhBMP-2-treated C3H10T1/2 cells might result from the simultaneous presence of chondrocytes in these cultures, which was suggested by detectable levels of collagen type II mRNA (not shown). Such a surge of N-cad may not occur in C2C12 cells because there is no chondrogenic transition under rhBMP-2 induction of osteogenesis in these cells. Thus, N-cad seems to be relatively stage-specific but tissue non-specific in determining the fate of mesenchymal cells.

On the other hand, we found consistent up-regulation of C11 in both C3H10T1/2 and C2C12 cells after rhBMP-2 stimulation. Although C11 expression occurs transiently in the cephalic mesoderm and then in the paraxial mesoderm of the trunk during early development, at later developmental stages a wide variety of mesenchymal tissue in both mesodermal and neural crest derivatives express C11 [Simonneau et al., 1995]. Thus, this cadherin has been considered crucial for mesenchymal organization and is found primarily in mesenchymal cells [Hoffmann and Balling, 1995; Simonneau et al., 1995], including bone marrow stromal cells from which osteoblasts derive in adult bone [Cheng et al., 1998]. Hence, up-regulation of C11 in immature mesenchymal cells under stimulation by osteogenic factors contrasts with its down-regulation when cells undergo adipogenic or myogenic differentiation. In fact, both N-cad and C11 rap-

idly decline when cells undergo adipogenesis, and mature adipocytes express none of the three mesenchymal cadherins we have studied. One can speculate that C11 and N-cad may allow sorting and segregation of the mesenchymal progenitors that are committing to osteogenic differentiation from those that are entering the adipogenic pathway. In the adult tissue, the capacity of bone marrow to generate precursors of bone-forming cells declines with age, while at the same time there is an increased prevalence of adipocytes. Therefore, regulated expression of C11 and N-cad may provide recognition signals governing the fate of immature bone marrow precursors, thereby contributing to the bone remodeling potential of the skeleton.

Preliminary reports suggest that mice genetically deficient in C11 have a mild but distinct skeletal phenotype, consisting of craniofacial changes and abnormal shape of long bones [Kawaguchi et al., 1999], whereas the N-cad null mutation is lethal at day 10 of gestation [Radice et al., 1997]. Our findings suggest that both N-cad and C11 may have some redundant roles in osteogenesis. N-cad is perhaps critical during the very early stages of differentiation and particularly during the condensation and development of the cartilaginous scaffolding of bone rudiments. N-cad expression clearly persists in mature and adult bone, as shown by its localization on cells lining the periostium and osteocytes in rat tibia by in situ hybridization [Ferrari et al., 2000], and by its presence in human and murine osteoblastic cells [Cheng et al., 1998]. C11, which has an increase in abundance during osteogenic differentiation, may have a more important function at later stages. Thus, the relative roles of these two cadherins in bone development and tissue function might be temporally different but functionally complementary.

In C2C12 cells, N-cad and C11 showed reciprocal changes in myogenic and osteogenic pathways. Our results are in agreement with those of Kawaguchi et al. [Kawaguchi et al., 1999], who observed M-cadherin switching to C11 when C2C12 cells were induced towards the osteogenic lineage by treatment with BMP-2. The same pattern of early transient expression of N-cad was also observed during myogenesis, another differentiation pathway in which N-cad-mediated cell-cell interactions are believed to be important, at least in the earlier stages. During embryonic development, N-cad expression is evident in limb buds as early as E6 and its expression is down-regulated as synaptogenesis and secondary myogenesis proceed. By E17, N-cad disappears in muscles [Cifuentes-Diaz et al., 1994]. Recent studies on N-cad null myoblasts demonstrated that N-cad is not strictly required for myoblast fusion [Charlton et al., 1997]. By contrast M-cadherin, considered a muscle-specific cadherin, is expressed in forelimb rudiments at E11.5 and persists until one week after birth [Moore and Walsh, 1993]. These data suggest that a similar paradigm may direct osteogenic and myogenic differentiation. In both lineages, N-cad seems to have a critical function during the early stages of cell commitment to each pathway, perhaps acting as a transitory switch, as in the case of muscle development. In mature tissues, coexpression of another cadherin —C11 for osteoblasts, M-cad for myoblasts — is required to define lineage specific features. This model might explain why C2C12 cells, which are already committed to become myoblasts, consistently express high levels of N-cad that are not up-regulated in response to rhBMP-2. Likewise, the apparent insensitivity of N-cad to rhBMP-2 in MC3T3-E1 cells may be explained by the fact that these cells, though immature, are already fully committed to the osteoblastic lineage.

R-cad, which was originally identified in chicken retina [Inuzuka et al., 1991], was later found in a variety of cells including human osteoblast and stromal cells [Cheng et al., 1998]. In this study, both C3H10T1/2 and C2C12 cells expressed R-cad, though at a low abundance relative to N-cad and C11, and its expression was decreased to undetectable levels in all differentiation pathways. It has been shown that R-cad is present and mediates cell-cell interactions during myogenesis [Rosenberg et al., 1997] and neuronal morphogenesis [Ganzler and Redies, 1995]. However, we could not find any detectable level of R-cad in C2C12 cells during myogenesis, and down-regulation of R-cad by rhBMP-2 was consistent with our previous observations in human osteoblasts [Cheng et al., 1998]. Thus, the role of R-cad for mesenchymal cell differentiation remains obscure.

In conclusion, we have found that the repertoire of various cadherins changes during osteogenic, adipogenic, and myogenic differenti-

ation from mesenchymal progenitor cells. Although neither N-cad nor C11 represent strict tissue-specific markers, the relative abundance of these mesenchymal cadherins seems to define lineage specific features, perhaps providing specific molecular signatures for cell aggregation and differentiation.

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