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ARTICLES

N-Cadherin Mediated Distribution of β-Catenin Alters MAP Kinase and BMP-2 Signaling on Chondrogenesis-Related Gene Expression

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Abstract We have examined the effect of calcium-dependent adhesion, mediated by N-cadherin, on cell signaling during chondrogenesis of multipotential embryonic mouse C3H10T1/2 cells. The activity of chondrogenic genes, type II collagen, aggrecan, and Sox9 were examined in monolayer (non-chondrogenic), and micromass (chondrogenic) cultures of parental C3H10T1/2 cells and altered C3H10T1/2 cell lines that express a dominant negative form of N-cadherin (Δ390-T1/2) or overexpress normal N-cadherin (MNCD2-T1/2). Our findings show that missexpression or inhibition of N-cadherin in C3H10T1/2 cells results in temporal and spatial changes in expression of the chondrogenic genes Sox9, aggrecan, and collagen type II. We have also analyzed activity of the serum response factor (SRF), a nuclear target of MAP kinase signaling implicated in chondrogenesis. In semi-confluent monolayer cultures (minimum cell–cell contact) of C3H10T1/2, MNCD2-T1/2, or Δ390-T1/2 cells, there was no significant change in the pattern of MAP kinase or bone morphogenetic protein-2 (BMP-2) regulation of SRF. However, in micromass cultures, the effect of MAP kinase and BMP-2 on SRF activity was proportional to the nuclear localization of β-catenin, a Wnt stabilized cytoplasmic factor that can associate with lymphoid enhancer-binding factor (LEF) to serve as a transcription factor. Our findings suggest that the extent of adherens junction formation mediated by N-cadherin can modulate the potential Wnt-induced nuclear activity of β-catenin. J. Cell. Biochem. 95: 53–63, 2005. Published 2005 Wiley-Liss, Inc.†

Key words: N-cadherin; chondrogenesis; C3H10T1/2; micromass; MAP kinase; BMP-2; β-catenin; aggrecan; type II collagen

For initiation of chondrogenesis during embryogenesis or induction in vitro, cellular condensation is a required step producing alterations in cell shape and existing cell signaling activities that in turn result in deposition of cartilage matrix. Although interference with cellular condensation has shown to reduce chondrogenesis, the precise molecular mechanisms are not well understood [Seghatoleslami and Kosher, 1996; Oberlender and Tuan, 2000]. A member of the classical cadherin family, N-cadherin, a single pass transmembrane glycoprotein with a molecular mass of 150 kD, is transiently upregulated and functions as a cell–cell adhesion molecule during chondrogenesis [Oberlender and Tuan, 1994; Oberlender and Tuan, 2000]. N-cadherin is one of several characterized classical cadherins [Takeichi et al., 1990; Gumbiner, 1996] which like non-classical cadherins contains several extracellular calcium binding domains that participate in calcium-mediated cell–cell adhesion. The presence of a single dimerization tripeptide domain (HAV; histidine, alanine, valine) distinguishes

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the classical cadherins from non-classical members of this family of calcium-mediated adhesion molecules. This unique extracellular domain enables cells of the same developmental fate to regroup and engage in a collective morphogenetic activity that results in homogeneous tissue formation and cell–cell communication [Takeichi, 1988].

The intracellular domain of cadherins interacts with a group of proteins known as catenins [α-catenin and the *armadillo* proteins β-catenin, plakoglobin, and p120: for reviews see Wheelock and Knudsen, 1991; Kemler, 1993; Cowin and Burke, 1996; Gumbiner, 1996]. The catenins link cadherin molecules to the actin cytoskeleton, which is required for functional adherens activity. Adherens formation has recently been shown to be regulated by the activity of small GTPases [Rho and Rac; Braga et al., 1997; Takaishi et al., 1997] and is strengthened by Ca^{++} mediated lateral aggregation of cadherins along the surface of the cell membrane [Nagafuchi and Takeichi, 1988; Nagafuchi and Takeichi, 1989; Knudsen et al., 1995; Rimm et al., 1995]. Furthermore, Noren et al. [2001] have reported that functional adherens junctions in turn regulate the activity of Rho family GTPases that are implicated in cytoskeletal activated cell signaling pathways involved in regulation of nuclear factors such as serum response factor (SRF) [Hill et al., 1995; Gineitis and Treisman, 2001], a major regulator of the *c-Fos* gene. The c-Fos protein is a major component of AP-1 transcription factor and both AP-1 and SRF transcription factors are also known targets of the mitogen activated protein kinase signaling cascade (MAP kinase).

While classical cadherins share a high degree of structural similarities, and interact with similar cytoplasmic proteins, cells genetically modified in expression of these adherens molecules acquire different behaviors. For example, forced expression or gain of function of N-cadherin in non-invasive, E-cadherin expressing human breast cancer cells results in cell motility; N-cadherin exerts a dominant effect over the activity of E-cadherin [Nieman et al., 1999; Hazan et al., 2000]. Therefore, differential distribution and association with intracellular signaling proteins places cadherin molecules in the category of important players involved in cell signaling and embryonic morphogenesis. In fact, close cell–cell contact involving cadherins is shown to play an important role in cellular differentiation and specific gene regulation [Redfield et al., 1997; Linask et al., 1998; Seghatoleslami et al., 2000; Goichberg et al., 2001]. In addition, linkage to the actin cytoskeleton and calcium-mediated lateral condensation of classical cadherins results in change of cellular shape, a morphological event that has been shown to play an important role in regulation of chondrogenesis [Zanetti and Solursh, 1984; Zanetti and Solursh, 1986]. In addition to the ubiquitously active MAP kinase cascade and changes in cellular shape, the signaling activities regulated by bone morphogenetic protein-2 (BMP-2) also play a crucial role in chondrogenesis [Zanetti and Solursh, 1984; Zanetti and Solursh, 1986; Denker et al., 1999; Oh et al., 2000; Seghatoleslami and Tuan, 2002].

In this work, we have used the multipotential embryonic mouse C3H10T1/2 cell line as a convenient source of cells that are amenable to genetic manipulation in order to study the role of N-cadherin in chondrogenesis specific cell signaling. The pioneering work by Taylor and Jones [1979] indicates that C3H10T1/2 cells, when maintained in appropriate culture conditions, can traverse multiple developmental pathways including myogenesis, adipogenesis, osteogenesis, and at a lower frequency of about 5%, chondrogenesis. Recent work from our laboratory indicates that chondrogenesis is favored when C3H10T1/2 cells are cultured as highly dense micromass cultures and exposed to BMP-2 [Denker et al., 1999]. Our findings also indicate that, in micromass cultures as compared with monolayer cultures of C3H10T1/2 cells, the activity of AP-1, a known target of the MAP kinase signaling cascade, is dramatically reduced and that this low level of AP-1 activity is maintained over an extended period of time coincident with progression of overt chondrogenesis [Seghatoleslami and Tuan, 2002]. Furthermore, interference with AP-1 activity in C3H10T1/2 micromass cultures resulted in reduced matrix sulfate incorporation [Seghatoleslami and Tuan, 2002], and similarly, overexpression of a component of AP-1, c-Fos, also resulted in inhibition of chondrogenesis in mouse sarcoma ATDC5 cells [Thomas et al., 2000]. Because of these findings, therefore, we have postulated that a steady low threshold level of AP-1 is required for optimal chondrogenesis and cartilage matrix deposition. Since cellular condensation, an initial stage of chondrogenesis, is associated with transient

expression of N-cadherin and change in cellular shape [Oberlender and Tuan, 1994; Haas and Tuan, 1999], we hypothesize that this calcium-mediated adherens molecule can play a role in alteration of the overall ubiquitous cell signaling activities such as that of MAP kinase and AP-1 that in turn are conducive to the initiation and maintenance of chondrogenesis.

To manipulate the activity of N-cadherin, we have created established C3H10T1/2 derivative cell lines that overexpress the normal or mutant form of N-cadherin. Previously, our laboratory has shown that anomalous expression and activity of this calcium-dependent adherens molecule results in abnormal sulfate incorporation and alcian blue staining of cartilage matrix within micromass cultures of C3H10T1/2 cells [Haas and Tuan, 1999]. To begin to understand the molecular mechanisms by which N-cadherin affects chondrogenesis, we have examined how perturbation of synthesis and function of N-cadherin affects the ubiquitous MAP kinase activity and also chondrogenic gene regulation and activity of the SRF transcription factor, an obligate regulator of the c-Fos component of the AP-1 heterodimer. We have also investigated the effect of N-cadherin function on BMP-2 signaling that has been shown to promote chondrogenesis in C3H101/2 cells [Denker et al., 1999]. In this article, we suggest that normal N-cadherin activity is required for proper temporal MAP kinase and BMP-2 regulation of chondrogenic genes such as, type II collagen, aggrecan, and Sox9. In addition, we show here that the changes in MAP kinase and BMP-2 activity in C3H10T1/2 cell lines with perturbed calcium-mediated adherence junctions result in alterations of SRF activity involved in the regulation of the *c-Fos* gene, whose product is suggested to play an important role in the regulation of chondrogenesis.

MATERIALS AND METHODS

Establishment of Cell Lines

The MNCD2-T1/2 cell line was established by transfection and genomic integration of the pBATMNC plasmid encoding, the mouse N-cadherin [Miyatani et al., 1989] and the Δ390-T1/2 cell line was constructed likewise using the pMiwc390Δ expression construct [Fujimori and Takeichi, 1993]. Both lines have been described previously [Haas and Tuan, 1999]. The MNCD2-T1/2 subline has been shown to express normal mouse N-cadherin at two to three times the level of the endogenous gene [previously defined as clone 1; Haas and Tuan, 1999]. The Δ390-T1/2 subline expresses a dominant negative mutant form of chicken N-cadherin that is missing the extracellular calcium binding domains including the dimerization HAV domain [Fujimori and Takeichi, 1993]. To generate cell lines containing the SRF-luciferase construct, C3H10T1/2, MNCD2-T1/2, and Δ390-T1/2 cells were cotransfected with a simple luciferase reporter construct driven by tandem repeats of SRF responsive elements fused with a TATA box (obtained from Stratagene) and a separate construct containing neomycin resistant gene. The established neomycin resistant colonies for each cell line were pooled together, expanded in culture, and designated as SRF-10T1/2, SRF-Δ390T1/2, or SRF-MNCD2T1/2. All cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin G (Sigma, St. Louis, MO), and 50 μg/ml streptomycin sulfate (Sigma). For induction of chondrogenesis, C3H10T1/2 cells and its sublines were cultured as micromass (1×10^5 cells/10 μl); and for non-chondrogenic control cultures, cells were plated in monolayer (1×10^5 cells seeded in 35 mm diameter culture dishes) and fed with Ham's F12 medium (Bio Whittaker, Walkersville, MD.) supplemented with 10% FBS and antibiotics. Micromass cultures treated with or without recombinant human bone morphogenetic protein-2 (BMP-2: 100 ng/ml) were also treated with the inhibitor of ERK1/2 (PD 98059; 5 μM; Calbiochem-Novabiochem Corp., San Diego, CA) or the inhibitor of P38 (SB 202190; 5 μM; Calbiochem-Novabiochem Corp.) MAP kinases.

Immunofluorescent Microscopy

C3H10T1/2 cell lines were seeded at a subconfluent density of 2.5×10^4 cells/chamber in four chambered glass slides and cultured for 24 h. Subconfluent cultures were washed with PBS and fixed in 2% paraformaldehyde/PBS for 10 min. Fixed slides were then washed in PBS followed by incubation in 0.15M glycine in PBS (twice, 10 min each). The cells were then washed in PBS followed by incubation in PBS containing 0.5% Triton X-100 twice, 5 min each time. Finally, after four PBS washes, the cells were incubated in blocking agent (10% goat serum in PBS) at room temperature for 60 min. Slides

were incubated with monoclonal antibody targeting the carboxy terminus of the 92 kD β-catenin (CAT-5H10; ZYMED, San Francisco, CA) for 1 h. After three washes, initially with 10% goat serum/PBS and then twice with PBS, slides were incubated with a FITC conjugated secondary antibody. Immunofluorescence imaging was done using confocal microscopy (Odyssey XL Confocal Laser Scanning Microscope).

Western Blot Analysis

Whole cell extracts were prepared from two plates each containing five micromass cultures at 5×10^5 cells/50 μl each. Nuclear extracts were prepared as described previously [Seghatoleslami and Tuan, 2002]. Whole cell extracts (10–20 μg) or the nuclear extracts (4 μg) were separated on SDS–PAGE gels and electrotransferred onto nitrocellulose blotting membranes. The blots were blocked in TBST (Tris-buffered saline, pH 7.4, 0.05% Tween 20) containing 3% BSA for 1 h followed by three 5 min washes in TBST and then incubated with TBST containing 0.05% BSA and the primary antibody (CAT-5H10) for 1 h. After three washes in TBST, 5 min each, the blots were incubated in horseradish peroxidase conjugated secondary antibody for 45 min followed by TBST washes and chemoluminesence detection using the ECL system (Amersham Biosciences, Piscataway, NJ). After detection of the β-catenin protein, the blot was stripped according to the ECL protocol provided by Amersham Biosciences and reprobed with the β-actin antibody (Santa Cruz, CA) as described above. The band intensities for β-catenin were normalized to the corresponding band intensities for β-actin in each sample. Quantification of the immunoreactive bands on the films were performed by optical scanning and densitometry using the Scion densitometry program (Scion Image for Windows version Beta 4.02).

RNA Extraction and RT-PCR

Five micromass cultures per treatment, with each micromass containing 5×10^5 cells/50 μl, were lysed and dissociated in 1 ml of Trizol reagent (Gibco BRL, Life Technologies, Grand Island, NY) and total RNA was isolated according to the manufacturer's protocol. Complementary DNA was reverse transcribed (RT) from 500 ng of total RNA using oligo dT primer and the Superscript™ First Strand Synthesis System for RT-PCR (Gibco BRL, Life Technologies). Specific cDNA amplifications were performed using PlatinumTaq DNA Polymerase enzyme (Gibco BRL, Life Technologies) and the following mouse-specific primer sets (forward/reverse): Sox9, TCCTAGTCTAGACACGCTCG-CGTG/GCTCTCGGCTCTCCGACTTCC; aggrecan, TTGCCAGGGGGAGTTGTATTC/GAC-AGTTCTCACGCCAGGTTTG; type II collagen, GTGAGCCATGATCCGC/GACCAGGATTTCC-AGG, and N-cadherin, CCACAGACATGGA-AGGCAATCC/CACTGATTCTGTATGCCGCA-TTC. The following primer set was used for amplification of the chicken N-cadherin expressed in Δ390-T1/2 cells: TGAAGGAAGCGGCT-CCACTG/TGGATCACTGATATTCCACGGAG. PCR band intensities were normalized to the density of the amplified bands obtained for the house keeping gene, β-actin, using the following primer set; GTGGGCCGCTCTAGGCACCAA/CTCTTTGATGTCACGCACGATTTC.

Luciferase Assay

Cultures were washed with cold PBS and scraped in 1× reporter lysis buffer (Promega), subjected to two freeze-thaw cycles, and the enzyme activity was assayed using the Luciferase Assay System kit from Promega. The light intensity generated was measured in 10 s intervals using a Monolight 2010 luminator (Analytical Luminescence Laboratory, San Diego, CA). Protein content of each sample was measured using the Bradford protein assay system (BioRad, Hercules, CA).

RESULTS

Perturbation of Normal N-Cadherin Function Results in Abnormal Regulation of Chondrogenesis in C3H10T1/2 Mesenchymal Cells

To disrupt N-cadherin mediated intercellular junction formation, we have utilized C3H10T1/2 cell lines overexpressing either normal [mouse; MNCD2; Miyatani et al., 1989] or the dominant negative form of N-cadherin [Δ390; a mutant form of chicken N-cadherin missing the extracellular calcium binding domains; Fujimori and Takeichi, 1993]. Using reverse transcriptase and PCR analyses, we have verified the exogenous expression of the normal and mutant N-cadherin in these cell lines; MND2-T1/2 cells express a twofold higher N-cadherin mRNA level than the parental

C3H10T1/2 cells and the Δ390-T1/2 cells overexpress the chicken N-cadherin transgene (Fig. 1D). Analysis of both cell lines in 2.5-day micromass and monolayer cultures indicated that N-cadherin could influence cellular signaling involved in progression of chondrogenesis. As shown in Figure 1, except the cultures treated with BMP-2 (Fig. 1C, lane 3), the activity of the type IIB isoform with respect to the type IIA isoform of collagen type II gene was upregulated in micromass cultures of Δ390-T1/2 cells (Fig. 1C, lanes 2, 4–7) compared to C3H10T1/2 cells cultured under identical conditions (compare with the corresponding bands in Fig. 1A). Overexpression of normal N-cadherin in MNCD2-T1/2 cells, on the other hand, resulted in inhibition of ERK1/2 and p38 MAP kinases and BMP-2 signaling evident by reduced activity of collagen type IIB, Sox9, and complete inhibition of aggrecan genes (Fig. 1; compare MNCD2-T1/2 RT-PCR panel with that of C3H10T1/2, lanes 2–6). Reduced activity of N-cadherin in Δ390-T1/2 cells, on the other hand, resulted in increase in the ratio of the type IIB/type IIA transcripts of collagen type II gene and increase in Sox9 gene expression in response to p38 inhibition (Fig. 1; compare lane 5 in Δ390-T1/2 panel with that of C3H10T1/2).

SRF Nuclear Factor in C3H10T1/2 Cells Is Responsive to Changes in N-Cadherin Function

To determine the cell signaling pathways affected by N-cadherin function, we have tested the activity of the SRF transcription factor in C3H10T1/2, Δ390-T1/2, or MNCD2-T1/2 cells that harbor a luciferase reporter construct driven by SRF regulatory elements (construction of these cell lines are described in the Materials and Methods). The SRF nuclear factor is an obligate transcriptional regulator of the *c-Fos* gene whose product is a component of the AP-1 heterodimer that binds to the

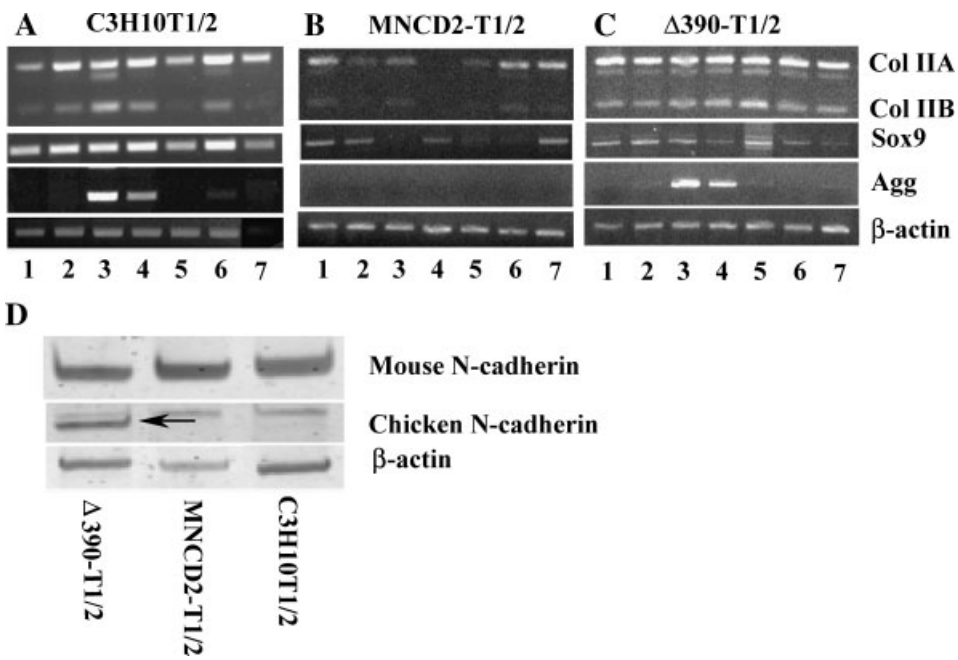

Fig. 1. Effect of perturbed N-cadherin expression on chondrogenesis of C3H10T1/2 mesenchymal cells. Gene expression was examined by RT-PCR analyses of type II collagen, aggrecan, and Sox9 in micromass cultures of C3H10T1/2 cells (**A**) or that of its cell line derivatives either overexpressing normal (**B**; MNCD2-T1/2) or a dominant negative form of N-cadherin (**C**; Δ390-T1/2). For each panel, **lanes 1–6** are micromass cultures, untreated or treated with PD 98059 (an ERK1/2 MAP kinase inhibitor), BMP-2, BMP-2 plus PD 98059, SB 202190 (a P38 MAP kinase inhibitor), and BMP-2 plus SB 202190, respectively. Activity of these genes in non-chondrogenic monolayer cultures is also shown for each cell line (**lane 7** on each panel). Amplification of the housekeeping-gene β-actin is used to normalize for differences in sample preparations and loading. RT-PCR analysis of the exogenous expression of the transgenes are also shown (**D**). The exogenous expression of the mutant form of the chicken N-cadherin (Δ390) was determined using primers specific for the chicken gene. Overexpression of the mouse N-cadherin was determined using primers specific to the mouse gene. Density of each band is normalized to the density of its corresponding β-actin band. MNCD2-T1/2 cells express twice the level of N-cadherin than that of the parental C3H10T1/2 cells. The experiments related to the pattern of aggrecan gene expression in the conditions shown above were performed at least twice.

promoter region of a number of chondrogenic genes. SRF is also a target of the MAP kinase signaling pathway that is involved in the regulation of chondrogenesis [Oh et al., 2000]. As shown in Figure 2A–C, the responsiveness of SRF in semi-confluent monolayer cultures (minimized cell contact) of the three SRF-cell lines in response to the inhibitors of ERK1/2 and p38 or to the same inhibitors in the presence of BMP-2 follows a similar pattern of modulation. However, in micromass cultures, inhibition of ERK1/2 MAP kinase with 5 μM PD 98059 resulted in upregulation of SRF activity in C3H10T1/2 and Δ390-T1/2 cells (SRF-T1/2 and SRF-Δ390T1/2 cells, respectively), and no change in the activity of this transcription factor in C3H10T1/2 cells overexpressing the normal N-cadherin (SRF-MNCD2T1/2 cells) (see Fig. 2D–F). Treatment with 5 μM SB 202190 (an inhibitor of p38 MAP kinase) resulted in no change in the activity of SRF in micromass cultures of SRF-MNCD2T1/2 (Fig. 2D), whereas the activity of SRF in micomass cultures of SRF-10T1/2 cells was inhibited (Fig. 2F). In contrast, the activity of this transcription factor in SRF-Δ390T1/2 micromass cultures in response to SB 202190 was induced (Fig. 2E). The promoter of chondrogenesis, BMP-2, induced SRF activity in SRF-10T1/2 and SRF-Δ390T1/2 but not in SRF-MNCD2T1/2 micromass cultures (Fig. 2D–F). When PD 98059 was added, BMP-2 treatment resulted in an induced activity of SRF-luciferase construct in micromass cultures of all these lines (Fig. 2D–F). For SRF-MNCD2T1/2 micromass cultures, this combination of BMP-2 and PD 98059 inhibitor was the only tested treatment that induced SRF-luciferase activity over that of control.

Changes in Levels and Cellular Distribution of β-Catenin in C3H10T1/2 Cells With Altered N-Cadherin Function

The formation of adherens junctions in the three C3H10T1/2 cell lines was examined by immunofluorescence microscopy to localize the submembranous as well as nuclear distribution of β-catenin protein using a mouse-specific monoclonal β-catenin antibody. The localization of β-catenin in the submembranous region is used as an indicator for the extent of functional calcium-mediated adherens junction formation. For the purpose of obtaining a greater cellular resolution, the distribution of β-catenin in the three cell lines was examined in subconfluent cultures. The biochemical distribution of β-catenin in micromass cultures was examined by cell fractionation followed by SDS–PAGE analysis. As shown in Figure 3, a weakly fluorescent but contiguous line of β-catenin was localized at the junction of the contacting C3H10T1/2 cells (Fig. 3B). In contrast, the MNCD2-T1/2 cell line that overexpressed exogenous mouse N-cadherin, displayed a much brighter and thicker fluorescent lining of β-catenin that was localized in between cells undergoing extensive cell–cell contact (Fig. 3A). However, Δ390-T1/2 cells expressing a dominant negative form of chicken N-cadherin exhibited less adherens junction formation than parental C3H10T1/2 cells, as evidenced by non-continuous β-catenin fluorescence labeling at the cell junctions (Fig. 3C). Changes in N-cadherin levels and function in C3H10T1/2 cells also resulted in differential cytoplasmic as well as nuclear distribution of β-catenin. In general, we found that if less β-catenin was localized to submembranous sites, more of this protein would move to the cytoplasm and nucleus (Fig. 3A–C). SDS–PAGE analysis of total and nuclear extracts isolated from micromass cultures of the above cell lines also revealed an interesting pattern of β-catenin distribution. A higher total content of β-catenin was quantified in parental C3H10T1/2 cells compared to either MNCD2-T1/2 or Δ390-T1/2 cells (Fig. 4A). Nuclear levels of β-catenin, on the other hand, were highest for Δ390-T1/2 cells followed by C3H10T1/2 and MNCD2-T1/2 cells (Fig. 4B). Interestingly, this pattern of β-catenin distribution correlates with the chondrogenic gene regulation such that the more β-catenin is localized to the nucleus (MNCD2-T1/2 followed by C3H10T1/2 and then followed by Δ390-T1/2 cells) the more fluctuation in the signaling activities involved in regulation of chondrogenic genes as well as that of SRF nuclear factor (refer to Figs. 2D–F and 1A–C).

DISCUSSION

Formation of functional adherens junctions involving classical cadherins such as N-cadherin is dependent on their calcium-mediated lateral aggregation. Interaction of N-cadherin with intracellular catenins and cytoskeletal elements during calcium-mediated adherens junction formation can alter cellular signaling involved in specific gene regulation and cellular

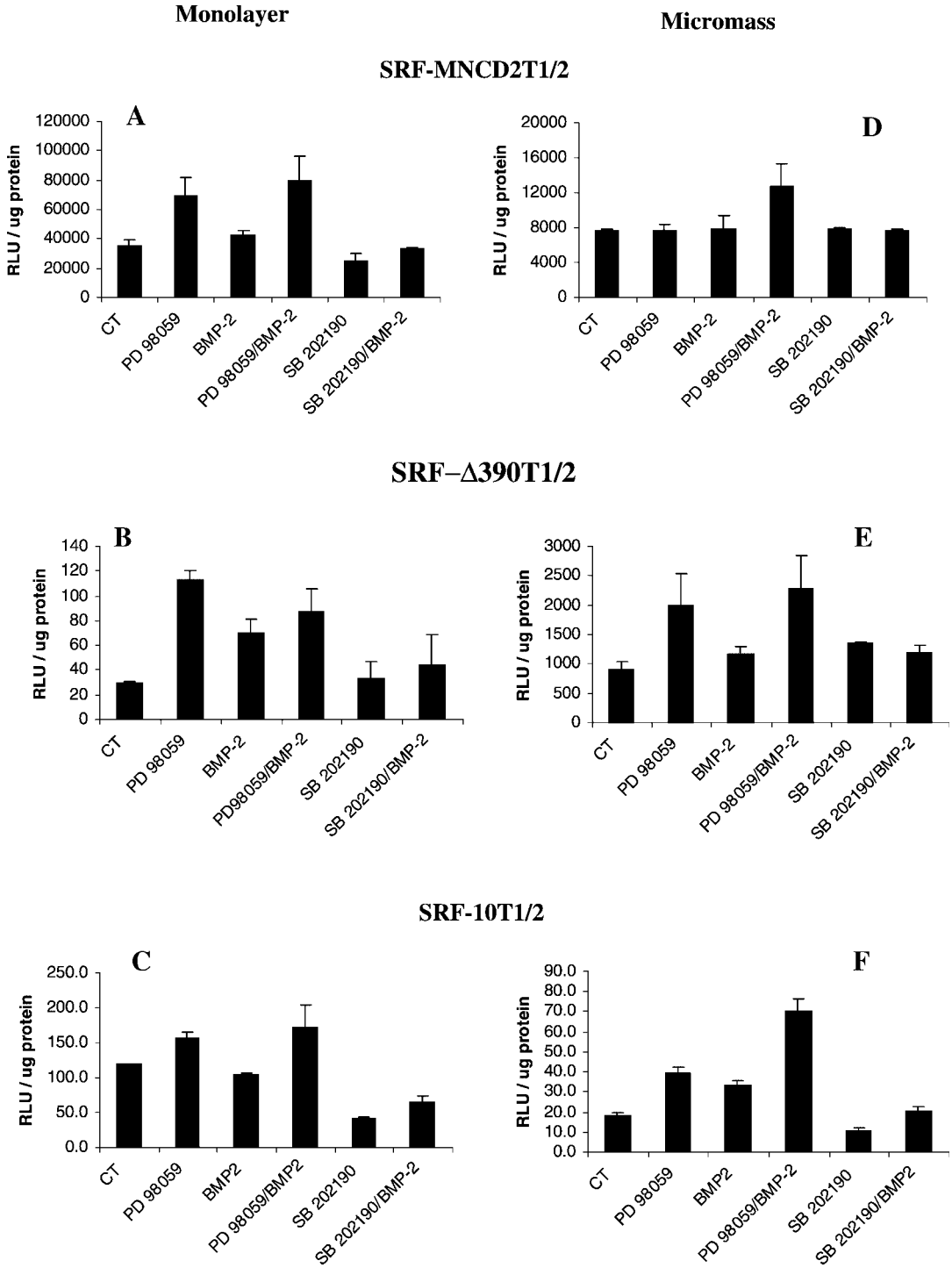

Fig. 2. Effect of abnormal function of N-cadherin on SRF activity in C3H10T1/2 cells. Effect of PD 98059 or SB 202190 on expression of SRF in monolayer or micromass cultures of the parental C3H10T1/2 cell line (SRF-10T1/2; **C** and **F**) and C3H10T1/2 cell lines overexpressing normal (SRF-MNCD2T1/2; **A** and **D**) or the dominant negative form of N-cadherin (SRF-Δ390T1/2; **B** and **E**). In addition to the inhibitors, the cultures were also treated with or without BMP-2. The establishment of the SRF cell lines is described in Materials and Methods. Luciferase activities are normalized to the protein content of each sample. The data represents the average of duplicate experiments ±SD.

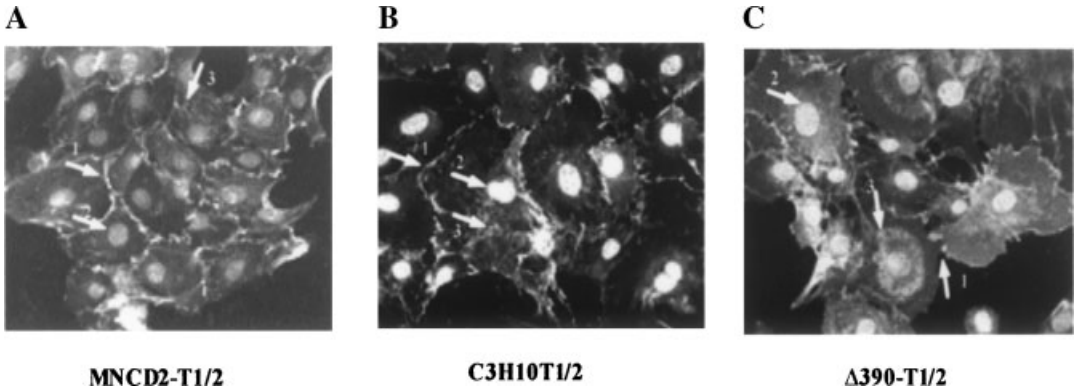

Fig. 3. Pattern of β-catenin distribution in the C3H10T1/2 cell lines with normal or abnormal N-cadherin function. Immunofluorescent detection of β-catenin in normal C3H10T1/2 cells (**B**) or derived cell lines of C3H10T1/2 overexpressing normal N-cadherin (**A**; MNCD2-T1/2) or a dominant negative form of N-cadherin (**C**; Δ390-T1/2). Arrows in each panel indicate the submembranous (arrow number 1), nuclear (arrow number 2), and cytoplasmic (arrow number 3) localization of β-catenin. The images were taken by laser confocal microscopy of cells grown at 80%–90% confluence on glass slides. Magnifications correspond to 200×.

differentiation [Redfield et al., 1997; Seghatoleslami et al., 2000; Goichberg et al., 2001]. In this work, we have extended these findings by investigating the mechanism by which classical cadherins such as N-cadherin can induce changes in cell signaling activities involved in cellular differentiation. To this end, we have studied chondrogenesis in C3H10T1/2 cells in which changes in MAP kinase activity, BMP-2 signaling, and cytoskeletal organization, can be linked to N-cadherin levels and functions. Specifically, our approach was to investigate

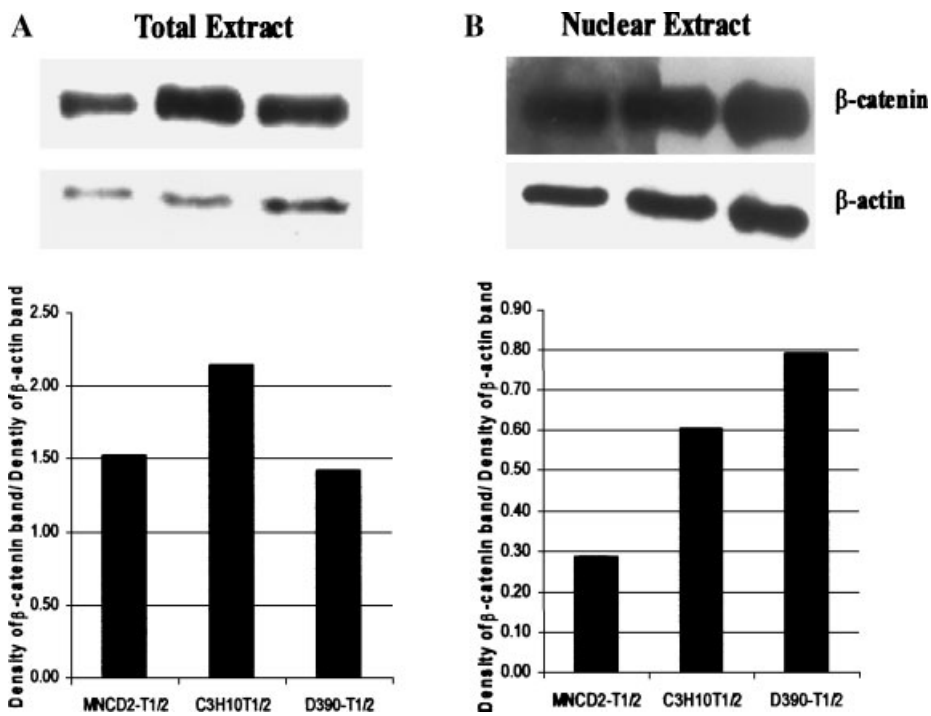

Fig. 4. Western blot analysis of total and nuclear levels of β-catenin in micromass cultures of C3H10T1/2 cell lines with perturbed N-cadherin function. **Panel A**: Total levels of β-catenin in normal C3H10T1/2 (**lane 2**) and its derived cell lines either overexpressing normal mouse N-cadherin (MNCD2-T1/2; **lane 1**) or a dominant negative form of chicken N-cadherin (Δ390-T1/2; **lane 3**). **Panel B**: It shows the nuclear content of β-catenin in identical cultures as shown in panel A. Histograms below indicate the densitometric comparison of the total or nuclear content of β-catenin in the micromass cultures of the three cell lines of C3H10T1/2 normalized to β-actin in each sample. These experiments were performed two times.

the effect of modulating the degree of functional adherens junction formation involving N-cadherin on chondrogenic gene regulation and MAP kinase signaling. Both overexpression of normal N-cadherin (to increase functional calcium-mediated adhesion) or a defective form of N-cadherin that cannot form calcium-mediated cell–cell interactions resulted in alterations in chondrogenic gene regulation, which was concomitant with alterations in BMP-2 and MAP kinase signaling activities as well as cellular redistribution of stable β-catenin. Quantification of total and nuclear β-catenin protein in micromass cultures of the three cell lines suggested a direct relationship between the nuclear β-catenin levels and the activity of BMP-2 and MAP kinase signaling and chondrogenic gene regulation. In Δ390-T1/2 cells, with less functional N-cadherin mediated adherens junctions, a higher nuclear content of β-catenin in comparison to normal C3H10T1/2 cells was observed. In micromass cultures of this cell line, while expression of chondrogensis related genes was not significantly affected, a stably incorporated SRF-luciferase construct (SRF-Δ390T1/2) was more responsive to the changes in the activity of ERK1/2, p38, or BMP-2 signaling than in normal SRF-T1/2 micromass cultures. The pattern of the activity of SRF-luciferase construct in both C3H10T1/2 and Δ390-T1/2 cells indicated an additive effect for ERK inhibition and BMP-2 treatment suggesting the involvement of the two distinct Smad and MAP kinase activity in chondrogenesis. On the other hand, as compared with C3H10T1/2 cells, cells overexpressing normal N-cadherin (MNCD2-T1/2) in contacting monolayer cultures revealed a much lower nuclear but higher submembranous fluorescent localization of β-catenin. In contrast to the micromass cultures of C3H10T1/2 cells, the response of an SRF-luciferase reporter construct to ERK1/2, p38, and BMP-2 in micromass cultures of MNCD2-T1/2 cells was greatly compromised (Fig. 2). Reduced nuclear localization of β-catenin in MNCD2-T1/2 cells seeded in micromass cultures was confirmed by Western blot analysis and was associated with complete inhibition of aggrecan and reduced type II collagen and Sox9 gene expression. Our findings are in line with the findings of Oh et al. [2000] that also linked reduced chondrogenesis with increased N-cadherin expression. Interestingly, the pattern of the response of SRF-luciferase construct, in the non-contacting monolayer cultures (a culture condition with minimized adherens junction formation) of the three cell lines, to the various stimuli mentioned above was similar. Therefore, in chondrogenic cultures of C3H10T1/2 cells (high density micromass cultures, a culture condition promoting maximum adherens junction formation), elevated or abnormal formation of N-cadherin mediated adherens junction results in changes in submembranous as well as nuclear localization of β-catenin that is concomitant with alterations in the signaling mechanisms known to regulate chondrogenesis. In contrast to our findings, results reported by Goichberg et al. [2001], indicate that the increased formation of N-cadherin mediated adherens junctions and reduced nuclear levels of β-catenin result in increased myogenesis as evidenced by myogenin gene expression and myotube formation [Goichberg et al., 2001]. This disparity suggests that the effect of adherens junctions on cell signaling and cellular differentiation is dependent on the cell type and the molecular context of the cells under study. In embryonic C3H10T1/2 cells therefore, an increase in both submembranous and nuclear localization of β-catenin is conducive to initiation and progression of chondrogenesis. Whether myogenesis of C3H10T1/2 cells requires reduced nuclear localization of β-catenin is not known. Our findings also indicate that in addition to the involvement of β-catenin, N-cadherin mediated regulation of chondrogenesis induced by BMP-2 involves modulation of signaling by the mitogen activated protein kinases, ERK1/2, and the stress activated MAP kinase, P38. Whether there is a direct interaction between the β-catenin signaling pathways and that of the BMP-2 and MAP kinase is the subject of further studies.

Our findings further indicate that although as shown by Oh et al. [2000], ERK1/2 and P38 MAP kinases might have an opposing effect on the overall process of chondrogenesis in the chick limb mesenchymal system, with regard to aggrecan gene expression in C3H10T1/2 cells this effect was not observed. Inhibition of ERK1/2 did not result in upregulation of aggrecan gene expression in untreated C3H10T1/2 micromass cultures (Fig. 1A, lanes 1 and 2) but rather resulted in reduced effect of BMP-2 on the expression of this gene (Fig. 1A, lanes 3 and 4). Inhibition of P38 MAP kinase, on the other hand, resulted in total inhibition of BMP-2 effect on aggrecan gene expression (Fig. 1A,

lanes 5 and 6). These findings therefore suggest that ERK1/2 and p38 MAP kinases have a strong positive effect on BMP-2 regulation of aggrecan gene expression in C3H10T1/2 cells. Whether during chondrogenesis of C3H10T1/2 cells there is a direct interaction between BMP-2 and MAP kinase pathways involving ERK1/2 and p38 is the subject of future studies.

The immunofluorescence analysis of the contacting monolayer cells and Western analysis of the micromass cultures suggest that in C3H10T1/2 cells there is an inverse relationship between the levels of functional N-cadherin-mediated adherens junction formation and the nuclear content of β-catenin. Increased stability of β-catenin resulting in cytoplasmic as well as nuclear accumulation of this factor in Δ390-T1/2 cells also suggested the involvement of a Wnt like signaling activity, which is known to play a major role in regulation of chondrogenesis [Fischer et al., 2002]. Therefore, N-cadherin mediated redistribution of β-catenin appears to be a mechanism by which Wnt-mediated chondrogenesis can be regulated. Furthermore, our studies strongly suggest that modulation of MAP kinase and BMP-2 signaling are mechanisms by which β-catenin/lymphoid enhancer-binding factor (LEF) complexes can regulate chondrogenesis related gene expression.

Another interesting observation in this study is the pattern of cytoplasmic distribution of β-catenin in C3H10T1/2 cells that contrasts with that observed in the Δ390-T1/2 cell line. In parental C3H10T1/2 cells, β-catenin is not widely distributed but instead is directed along the length of the polar actin cytoskeleton towards the nucleus. In Δ390-T1/2 cells, however, cytoplasmic β-catenin is more evenly distributed around the nucleus possibly because of inadequate actin cytoskeletal organization due to abnormal adherens junction formation. We propose that the actin cytoskeleton and its associate proteins might play a role in allowance of efficient nuclear transport or cytoplasmic stabilization of β-catenin. This proposal is consistent with the report of Rosin-Arbesfeld et al. [2001], on actin-dependent transport and association of adenomatous polyposis coli (APC), a component of Wnt signaling pathway that regulates the stability of β-catenin, to the cell membrane. It will be of great interest to determine whether as compared with C3H10T1/2 cells there is a difference in the level and membrane localization of APC in Δ390-T1/2.

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