Journal of Cellular Biochemistry 77:252–264 (2000)

Upregulation of Myogenin by N-Cadherin Adhesion in Three-Dimensional Cultures of Skeletal Myogenic BHK Cells

M. Reza Seghatoleslami, Linda Myers, and Karen A. Knudsen*

Lankenau Medical Research Center, Wynnewood, Pennsylvania 19096

Abstract Cells of the baby hamster kidney (BHK) line express the skeletal muscle determining transcription factor MyoD but fail to differentiate. Unlike most skeletal myogenic cells, which express multiple members of the cadherin family of cell-cell adhesion proteins, the BHK cells lack a robust cadherin adhesion system. We previously published that forced expression of N- (or E)-cadherin in BHK cells increases the level of endogenous catenins, mediates strong cell-cell adhesion, and enhances differentiation of BHK cells induced to differentiate by placing them in three-dimensional (3-D) culture (Redfield et al. [1997] J. Cell. Biol. 138:1323–1331). This report demonstrates that N-cadherin adhesion upregulates the protein level of nuclear myogenin in cells induced to differentiate by 3-D culture. Myogenin is a transcription factor required for differentiation of skeletal muscle. It was not detected in monolayer culture, whether the cells expressed N-cadherin or not, nor was it upregulated in 3-D cultures of cells lacking N-cadherin. The activity of two myogenin-chloramphenicol acetyltransferase (CAT) reporter constructs containing 3.7 or 1.1 kb upstream regulatory region of the mouse myogenin gene was increased significantly in N-cadherin-expressing cells induced to differentiate by 3-D culture. Our observations indicate that N-cadherin adhesion stimulates skeletal myogenesis by upregulating myogenin. J. Cell. Biochem. 77:252–264, 2000.

Key words: cadherins; cell-cell adhesion; myogenin; skeletal myogenesis

Skeletal myogenesis is a multi-step process that is temporally and spatially regulated in the embryo [reviewed by Olsen, 1992; Emerson, 1993; Lassar and Munsterberg, 1994, Buckingham, 1995; Cossu et al., 1996; Yun and Wold, 1996]. Execution of the skeletal muscle program involves coordinate regulation of cell cycle progression and specific gene expression, culminating in the appropriate number of muscle cells, as well as their correctly timed determination and differentiation. Members of a family of basic helix-loop-helix transcription factors, including MyoD, myf 5, myogenin, and MRF4, are essential for skeletal myogenesis [reviewed by Weintraub, 1993; Rudnicki and Jaenisch, 1995, Ludolph and Konieczy, 1995]. In vitro, exogenous expression of each transcription factor promotes skeletal muscle differentiation efficiently in cells of mesenchymal origin [Davis et al., 1987; Choi et al., 1990; Auradé et al., 1994]. Deletion of individual myogenic transcription factors in mice has shown that MyoD or myf5 can induce commitment of precursor cells to the skeletal muscle lineage. By contrast, myogenin plays a unique role and is required for the differentiation of cells committed to becoming skeletal muscle [see Megeney and Rudnicki, 1995; Arnold and Braun, 1996; Olson et al, 1996]. Although considerable information exists on the mechanisms by which skeletal myogenic transcription factors affect expression of muscle specific genes, less is known about the molecular events regulating expression of the myogenic transcription factors themselves.

Cellular interactions mediated by members of the cadherin family of Ca^{2+}-dependent cell-

Grant sponsor: LMRC; Grant sponsor: Lankenau Foundation.

M. Reza Seghatoleslami is currently at the Department of Orthopaedic Surgery, Thomas Jefferson University, Orthopaedic Research Laboratory, 501 Curtis Building, 1015 Walnut Street, Philadelphia, PA 19107.

*Correspondence to: Karen A. Knudsen, Lankenau Medical Research Center, 100 Lancaster Avenue, Wynnewood, PA 19096. E-mail: knudsenk@mlhs.org

Received 15 September 1999; Accepted 3 November 1999

cell adhesion proteins have been shown to play important roles throughout myogenesis [reviewed by Knudsen and Horwitz, 1994; McDonald et al., 1995]. Developing skeletal muscle expresses multiple cadherins, including N-, M-, R-, and T-cadherin, and cadherin-11 [Hatta and Takeichi, 1986; Donalies et al., 1991; Inuzuka et al., 1991; Fredette and Ranscht, 1994; Kimura et al., 1995]. This finding is in contrast to cardiac muscle, which expresses predominantly N-cadherin [Radice et al., 1997]. Cadherin adhesion has been implicated in a variety of cellular events during cardiac and skeletal myogenesis, including cell-cell recognition and adhesion, cell migration, skeletal myoblast fusion, cytoskeleton organization, contact inhibition of growth, somite and heart morphogenesis in embryos, and differentiation of cardiac and skeletal muscle [Knudsen et al., 1990; Mege et al., 1992; Holt et al., 1994; Zeschnigk et al., 1995; Brand-Saberi et al., 1996; Linask et al., 1997, 1998; Rosenberg et al., 1997; Radice et al., 1997; George-Weinstein et al., 1997; Imanaka-Yoshida et al., 1998; Huttenlocher et al., 1998]. In addition to its well-characterized structural role, cadherin adhesion initiates intracellular signaling events required for the progression of myogenesis [George-Weinstein et al., 1997; Redfield et al., 1997; Linask et al., 1997; Imanaka-Yoshida et al., 1998].

Cadherins form a family of cell-cell adhesion proteins that serve as the core component of the adherens junction [reviewed by Yap et al., 1997]. Classical cadherins such as N-, E-, and P-cadherin are single-pass transmembrane glycoproteins [see Takeichi, 1990; Grunwald, 1993, 1996; Gumbiner, 1996]. The extracellular domains of identical cadherin family members interact in a trans-homotypic manner to bring about cell recognition and adhesion. Cadherin dimerization, linkage to the actin cytoskeleton via α- and β-catenin, and their organization into adherens junctions all act to strengthen cell-cell adhesion [see Tsukita et al., 1992; Wheelock et al., 1996]. The strength of adhesion is regulated by proteins such as p120 catenin ($p120^{ctn}$), as well as by kinases, phosphatases, and auxiliary proteins that probably vary with cell type [see Tonks and Neel, 1996; Daniel and Reynolds, 1997]. In addition to playing an important role in cell adhesion, β-catenin functions in a cadherin-independent manner as an integral component of the Wnt/Wng signaling pathway and, as such, acts as a signaling molecule [see Peifer, 1995; Orsulic and Peifer, 1996; Miller and Moon, 1996; Cadigan and Nusse, 1997]. When complexed with transcription factors in the TCF/LEF family, β-catenin affects gene transcription and thereby alters cell properties such as growth and differentiation [Kühl and Wedlich, 1997; Bienz, 1998].

The mechanism by which cadherins mediate cell-cell interactions is well understood, as is their linkage to catenins and the actin cytoskeleton. In addition, the role of β-catenin as a signaling molecule is being elucidated. By contrast, less is known about signaling downstream of cadherin adhesion and its effect on gene transcription [see Fagotto and Gumbiner, 1996; Aplin et al., 1998]. To increase our understanding of how cadherin adhesion promotes skeletal myogenesis, we identified a cadherin-negative myogenic cell line (i.e., BHK) that permits manipulation of cadherin expression and activity. Manipulation of cadherin activity is problematic in myogenic cell lines, and also in vivo, because developing skeletal muscle normally expresses multiple cadherins.

The baby hamster kidney (BHK) cell line expresses the skeletal myogenic determining transcription factor MyoD and the myogenic enhancing transcription factor MEF2 [Redfield et al., 1997], and thus can be characterized as skeletal myogenic. However, in contrast to normal skeletal myogenic cells, which express cadherins, the BHK cells fail to express a robust cadherin cell-cell adhesion system [Redfield et al., 1997]. Moreover, they fail to differentiate [Redfield et al., 1997]. In many ways, the cells resemble rhabdomyosarcoma tumor cells, which express MyoD but continue to proliferate, fail to differentiate, and exhibit aberrant cell-cell adhesion [Peralta Soler et al., 1993].

We previously showed that forced expression of N- (or E)-cadherin stimulates the BHK cells to express sarcomeric myosin under certain culture conditions, indicating that cadherin adhesion is required for differentiation [Redfield et al., 1997]. Differentiation occurred only when the N-cadherin expressing cells were placed in three-dimensional (3-D) culture [Redfield et al., 1997]. The requirement for special culture conditions to initiate muscle differentiation is not unique to the BHK cell line. The

C2C12 satellite skeletal muscle cell line is stimulated to differentiate by allowing cells to reach confluence and then switching the medium from one containing 10–20% fetal bovine serum (FBS) to one containing 2% horse serum. 3-D culture is required to induce skeletal muscle differentiation of embryonic stem (ES) cells and P19 teratocarcinoma cells, even those forced to express MyoD [Skerjanc et al., 1994]. It is likely that the reduction in serum in the case of C2C12 cells, and the loss of cell-matrix adhesion in the case of BHK, ES, and P19 cells, reduces signals that stimulate proliferation and simultaneously suppress differentiation.

In this article, we show that N-cadherin adhesion upregulates the protein level of the nuclear myogenic transcription factor myogenin in BHK cells induced to differentiate. Myogenin was not detected in monolayer cultures of proliferating BHK cells with or without N-cadherin, nor was it upregulated in non-cadherin-expressing control cells induced to differentiate. We also detected a significant increase in the activity of myogenin-CAT reporter constructs in N-cadherin-expressing BHK cells induced to differentiate. Our data indicate that N-cadherin adhesion stimulates skeletal myogenesis by upregulating myogenin expression.

MATERIALS AND METHODS

Cell Culture

Four stably transfected cell sublines derived from the parent BHK cell line as described by Redfield et al. [1997] were used in the studies reported in this article. They include two independent control transfected lines carrying the puromycin resistance gene (Pac, clones 5 and 6), and two independent cloned lines transfected with the full-length chicken N-cadherin cDNA, and stably expressing the N-cadherin protein (N-cad; clones 21 and 24). N-cadherin expression is constitutive and controlled by the human β-actin promoter [Redfield et al., 1997]. The two N-cadherin-expressing clones exhibited similar behavior, as did the two control clones; thus, the differences between control and N-cadherin expressing BHK cells are not due to clonal variability. The data reported in this article are from only one set of clones (i.e., pac5 and N-cad 21). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 7% FBS and 15 mM Hepes. When cultured as subconfluent monolayers, the control BHK cells and the N-cadherin expressing cells proliferate at a similar rate, and neither cell line differentiates [Redfield et al., 1997]. To induce myogenic differentiation, the cells were cultured as 3-D aggregates in suspension as described [Redfield et al., 1997].

Antibodies

Sarcomeric myosin was detected with the MF20 mouse monoclonal antibody to chicken pectoralis myosin (Developmental Studies Hybridoma Bank) [Bader et al., 1982]. N-cadherin was detected with 6B3 [George-Weinstein et al., 1997] or 13A9 [Knudsen et al., 1995], and β-catenin was detected with 15B8 [Johnson et al., 1993]. A mouse monoclonal antibody detecting all isoforms of the p120 catenin ($p120^{ctn}$) (pp120) was purchased from Transduction Laboratories (Lexington, KY). Myogenin was detected with the F5D mouse monoclonal antibody from Developmental Studies Hybridoma Bank [Wright et al., 1991].

Immunofluoresence Light Microscopy

For detecting N-cadherin, β-catenin, $p120^{ctn}$, and myogenin in monolayer cultures, the cells were grown on 18-well slides for 72 h. Subsequently, the cells were fixed with either ice-cold methanol or 2% paraformaldehyde followed by 0.5% Triton X-100. For detecting proteins in cells cultured as 3-D aggregates in suspension, the cells were collected by pipette and allowed to settle to the bottom of a centrifuge tube. The medium was removed completely, and the cell aggregates were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were washed with a 0.15 M glycine/0.5% Triton X-100/PBS solution. After removal of the wash buffer, the cell pellet was frozen on dry ice, placed into tissue-embedding medium (Tissue-Tek O.C.T. Compound, Miles, Elkhart, IN) on dry ice, and stored at −70°C; 5-μm-thick sections were cut using a cryostat, and the sections were mounted on a slide for staining. Antigens in fixed and permeabilized cells were detected with protein-specific primary monoclonal antibodies and a secondary CY3-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA). The immunofluorescence signal was detected

using a Zeiss Axiophot phase-contrast/epifluorescence microscope and the 40× objective, and recorded using Polaroid type 57 (ASA 3000) film.

Western Blot Analysis

Cell extraction and immunoblot analysis were performed according to Redfield et al. [1997] and Knudsen et al. [1995]. Briefly, cells were washed and extracted in a 10-fold excess of 1× sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris, pH 6.8, 20 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Insoluble material was removed by centrifugation. Protein concentration was determined by the Lowry assay. An equal amount of protein per lane was separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Blots were blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20. Proteins of interest were detected with primary mouse monoclonal antibodies followed by species-specific, alkaline phosphatase-conjugated secondary antibody (Fisher Scientific). Blots were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate substrates. Prestained molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, CA). Equal loading of samples was confirmed by blotting for actin using a monoclonal antibody (Sigma Chemical Co., St. Louis, MO).

DNA Constructs

The myogenin-CAT reporter constructs were a gift from Dr. Andres Buonnano (NIH, Bethesda, MD). Their construction and use in both in vivo and in vitro studies has been reported [Edmondson et al., 1992; Buonanno et al., 1993; Gibney and Buonanno, 1995]. Two myogenin-CAT reporter constructs were used in our studies. One, p3.7MygCAT, contains 3.7 kb of upstream region of the mouse myogenin gene (−3700 to +18 bp). The other, p1.1MygCAT, contains 1.1 kb (−1102 to +18 bp). Each upstream myogenin sequence was fused to the chloramphenicol acetyltransferase (CAT) reporter gene.

Transient Transfections

Transient transfection was carried out using LipofectAMINE Reagent (Gibco-BRL Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. For each transfection, 4 μg of DNA was used to transfect a 100-mm culture dish containing cells at 60–80% confluence; 24 h later, the cells from each plate were harvested with trypsin/EDTA and divided in half. One-half of the cells was subdivided again and re-plated in 60-mm culture dishes (2–4) at a concentration of 4×10^5 cells/60-mm dish. This culture condition is not permissive for differentiation, whether the cells express N-cadherin or not [Redfield et al., 1997]. The other half of the cells was subdivided identically and then placed in 3-D culture at a concentration of 3,000 cells/20 μl-hanging drop as described [Redfield et al., 1997]. This culture condition stimulates differentiation [Redfield et al., 1997]. Cells were maintained in monolayer or 3-D culture for an additional 60 h.

Chloramphenicol Acetyltransferase Assay

Cell monolayers were washed in PBS, and extracts for the CAT assay were prepared by first scraping cells in reporter lysis buffer (Promega, Madison, WI) and then performing two freeze-thaw cycles. Replicate 60-mm dishes (2–4) of monolayer cultures were handled separately. The identical number of replicate batches of cells in 3-D culture were collected by pipette, pelleted by low-speed centrifugation, washed in PBS, and extracted in reporter lysis buffer as above. Protein concentrations were determined for replicate cell extracts using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The two-phase partition method was used to assay CAT activity [Martin et al., 1990]. The samples were heated at 65°C for 15 min, to destroy endogenous acetyltransferase activity, and cell debris was removed by centrifugation at high-speed centrifugation. CAT activity was measured every 15 min for 1 h. The CAT activity value assigned to each sample was determined using linear regression analysis of the time-dependent increase in chloramphenicol acetylation. Values for CAT activity were adjusted to μg protein. Averages and standard deviations were calculated using the CAT/μg protein values for replicate samples. The averages for CAT/μg protein values were used to calculate the ratio of CAT activity for cells in 3-D culture versus monolayer culture, i.e., 3-D aggregate activity divided by monolayer activity. The CAT activity ratios

calculated from data collected in two to three independent experiments performed on separate days were averaged and the standard deviation calculated.

RESULTS

In a previous article by Redfield et al. [1997], we described in detail the BHK model system used in the present study. Briefly, BHK cells express the myogenic determining transcription factor MyoD, and thus are committed to the skeletal muscle lineage. However, they fail to differentiate, although they are capable of doing so [Redfield et al., 1997]. Two conditions must be met for the BHK cells to differentiate. First, they need a robust cadherin-mediated cell-cell adhesion, which we provided by forced expression of chicken N-cadherin, since the parent cells lack it [Redfield et al., 1997]. Second, the BHK cells need to be placed in 3-D culture for differentiation to occur. In the experiments described below, cells stably expressing chicken N-cadherin are compared with control transfected cells without N-cadherin, and proliferating monolayer cultures are compared with 3-D cultures.

We showed previously that forced expression of N-cadherin in BHK cells induces strong cell-cell adhesion and also upregulates the level of endogenous β-catenin, presumably by stabilizing the protein [Redfield et al., 1997] (Fig. 1). We found that increasing β-catenin through forced expression of an adhesion defective mutant cadherin does not support differentiation, indicating that cadherin adhesion is required [Redfield et al., 1997]. In the presence of full-length N-cadherin, the cellular localization of β-catenin shifts from the cytoplasm to the plasma membrane [Redfield et al., 1997] (Fig. 2). In a similar manner, N-cadherin expression also shifts the cellular localization of the p120 catenin ($p120^{ctn}$) to the plasma membrane (Fig. 2). However, in contrast to β-catenin, no increase in the total level of $p120^{ctn}$ accompanied N-cadherin expression (Fig. 1). Thus, the stability of $p120^{ctn}$ does not appear to be affected by the presence of a cadherin. In addition, our data suggest that when a cadherin is present, most of the $p120^{ctn}$ associates with it at the plasma membrane (Fig. 2).

It is interesting to note that the β-catenin level appears slightly higher in 3-D cultures, compared with monolayer cultures, whether

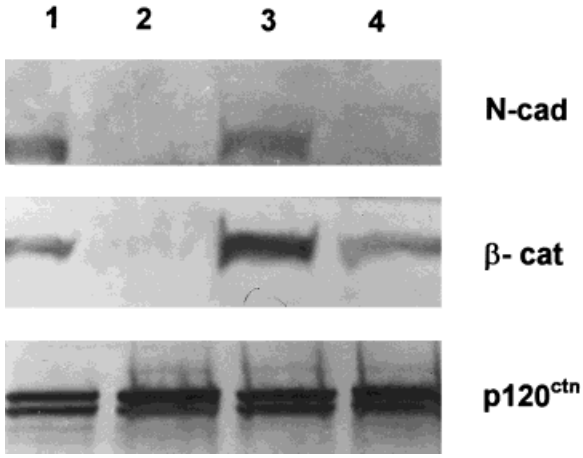

Fig. 1. Immunoblot analysis of N-cadherin, β-catenin, and p120 catenin ($p120^{ctn}$) in baby hamster kidney cells (BHK) cells lacking, or stably expressing, exogenous N-cadherin. BHK cells lacking exogenous N-cadherin (**lanes 2,4**), or cells stably expressing chicken N-cadherin (**lanes 1,3**) were generated as described [Redfield et al., 1997]. The cells were cultured either as monolayers attached to culture dishes (monolayer culture) (**lanes 1,2**) or as 3-D aggregates in suspension (3-D culture) (**lanes 3,4**). After 72 h, the cells were collected and extracted, and the protein concentration was determined. Equal amounts of protein were loaded in the lanes, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose. N-cadherin was detected with 13A9, β-catenin with 15B8, and $p120^{ctn}$ with pp120. Note that forced expression of N-cadherin upregulates β-catenin, but not $p120^{ctn}$. Note also that the β-catenin level appears higher in the 3-D aggregates compared with monolayer cells, regardless of whether the cells express N-cadherin. This appears to result from a slight upregulation of endogenous M-cadherin when the cells are placed in 3-D culture (data not shown).

the cells express exogenous N-cadherin or not (Fig. 1). This result suggested to us that the cells might upregulate an endogenous cadherin when they are placed in 3-D culture. Indeed, we detected a low level of M-cadherin in 3-D cultures of both control and N-cadherin-expressing BHK cells (data not shown). Expression of endogenous M-cadherin is consistent with the BHK cells being skeletal myogenic. However, the level of M-cadherin appears insufficient to promote significant differentiation [Redfield et al., 1997].

To begin to understand how cadherin adhesion enhances differentiation, we sought to assess the response of the myogenic transcription factor, myogenin, to cadherin stimulated differentiation of BHK cells. Previously we reported that neither the level nor phosphorylation of MyoD or MEF2 changed in response to cadherin adhesion [Redfield et al., 1997]. While

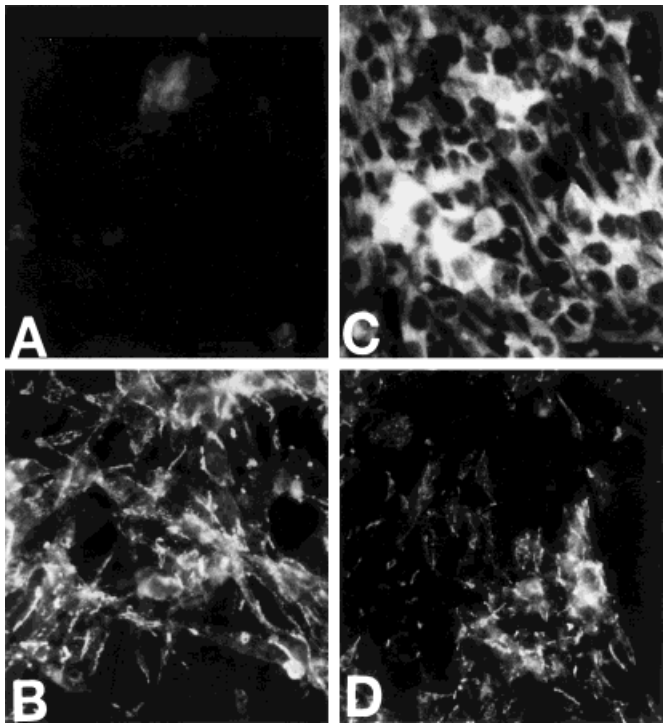

Fig. 2. Immunofluorescence localization of β-catenin and $p120^{ctn}$ in baby hamster kidney cells (BHK) cells with or without forced expression of N-cadherin. BHK cells without exogenous N-cadherin (**A,C**) or with exogenous N-cadherin (**B,D**) were grown for 48 h on 18-well slides. The cells were fixed for 10 min in ice cold methanol and then stained with either 15B8 to β-catenin (**A,B**) or pp120 to $p120^{ctn}$ (**C,D**), followed by CY3-conjugated goat anti-mouse IgG. Note that the presence of N-cadherin shifts the staining for both β-catenin (**B**) and $p120^{ctn}$ (**D**) to the plasma membrane. Immunoblot analysis in Fig. 1 shows that the presence of N-cadherin increases the total level of β-catenin, whereas there is no change in the total level of $p120^{ctn}$.

the action of Myf5 or MyoD is essential for committing cells to the skeletal muscle fate, myogenin is uniquely required for their differentiation [see Megeney and Rudnicki, 1995; Arnold and Braun, 1996; Olson et al., 1996]. Employing a specific monoclonal antibody (F5D) and immunofluorescence light microscopy, we detected a strong myogenin signal in the nuclei of N-cadherin expressing BHK cells induced to differentiate by 3-D culture (Fig. 3). The intensity of the myogenin signal varied among the cells, with 20–30% of the population being strongly positive. By contrast, myogenin staining was never detected in cells in monolayer culture, whether they expressed exogenous N-cadherin or not (not shown). Furthermore, only an occasional myogenin-positive cell was detected in 3-D cultures of control cells lacking exogenous N-cadherin (not shown). Attempts to quantify the results were not successful because the antibody failed to recognize denatured hamster myogenin. However, our results are similar to those of Goichberg and Geiger [1998], who used rat and mouse skeletal myogenic lines and a different experimental approach to show that N-cadherin adhesion upregulates myogenin.

To extend the above observation by our group and by Goichberg and Geiger [1998], we asked whether the upregulation in myogenin protein is attributable to increased transcription. The hamster myogenin cDNA has not been cloned. Therefore, we employed a full-length mouse myogenin cDNA to probe for endogenous myogenin mRNA in BHK cell extract by Northern analysis. However, use of the mouse cDNA produced equivocal data. As an alternative approach to evaluating myogenin transcription, we employed two myogenin-CAT reporter constructs bearing either 3.7 or 1.1 kb upstream region of the mouse myogenin gene (Fig. 4). These regulatory regions of the myogenin gene have been shown by other investigators to confer high

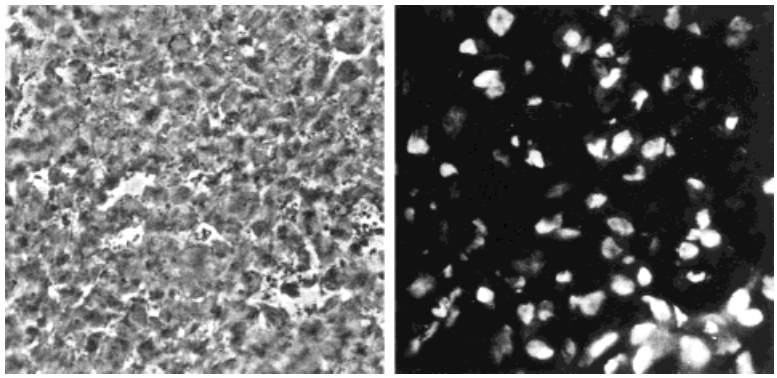

Fig. 3. Immunofluorescence detection of myogenin protein in N-cadherin-expressing baby hamster kidney cells (BHK) cells in 3-D culture. Control and N-cadherin-expressing cells were cultured as monolayers or aggregates in suspension (3-D culture); 72 h later, the monolayer cultures were fixed with paraformaldehyde, permeabilized with detergent, and stained for myogenin protein. The 3-D cell aggregates were collected with a pipette and allowed to settle in a centrifuge tube. Medium was removed, and the pellet was frozen and sectioned as if it were tissue before staining for myogenin. Myogenin staining was strikingly apparent in the nuclei of N-cadherin cells in 3-D culture (right). Phase contrast (left). Myogenin was never detected in cells in monolayer culture, regardless of whether they expressed N-cadherin (not shown). In addition, N-cadherin-negative control cells placed in 3-D culture exhibited only an occasional myogenin-positive cell (not shown). The fact that an occasional cell was positive for myogenin may reflect the activity of endogenous M-cadherin, which is upregulated to a low level when cells are placed in 3-D culture.

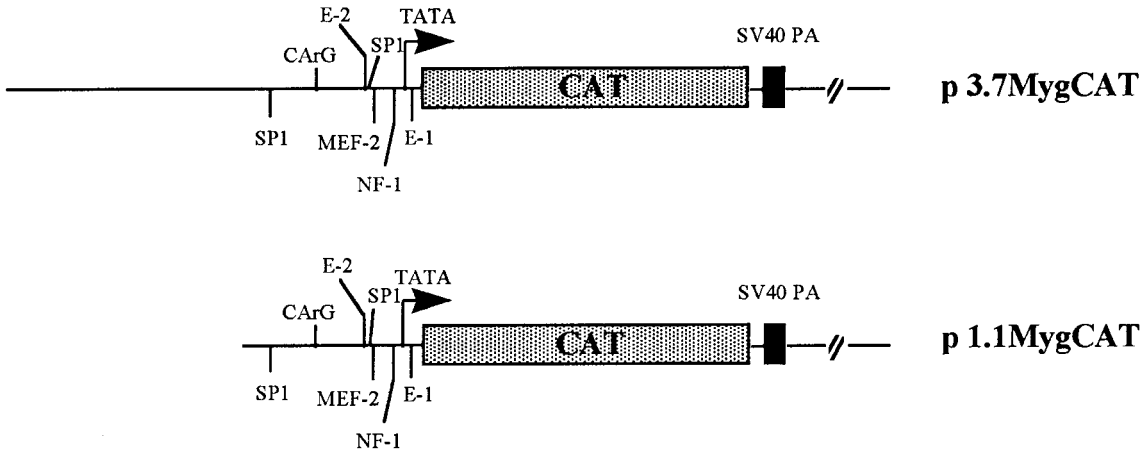

Fig. 4. Diagram of myogenin-chloramphenicol acetyltransferase (CAT) reporter constructs. The myogenin-CAT reporter constructs, p3.7MygCAT and p1.1MygCAT, were a gift of Dr. Andres Buonnano (NIH, Bethesda, MD) and have been described [Edmondson et al., 1992; Buonanno et al., 1993; Gibney and Buonanno, 1995].

level and tissue specific expression in mice and in mouse and chicken myogenic cells in vitro [Edmondson et al., 1992; Gibney and Buonanno, 1995]. Our experiments were conducted by transiently transfecting subconfluent monolayer cultures of BHK cells expressing or lacking exogenous N-cadherin with the p3.7MygCAT and p1.1MygCAT plasmids. Subsequently, CAT activity was analyzed in cells placed in 3-D culture (differentiation condition) versus monolayer culture (growth condition).

To interpret our CAT activity data correctly, it was necessary to control for differences in CAT activity due to variation in transfection efficiency between cells with or without N-cadherin, and between experiments performed on separate days. Therefore, we took the following approach (see also Materials and Methods). We transiently transfected mono-

TABLE I. CAT Activity for p3.7MygCAT and p1.1MygCAT Constructs*

Construct	Culture condition	Cell type	Average CAT activity/μg protein, ±SD (n = 2–4)		
			Exp 1	Exp 2	Exp 3
P3.7MgCAT	Aggregates	N-cad	2192.52 ± 1294.94	7345.96 ± 4555.20	2504.17 ± 73.36
		Control	1331.1 ± 54.28	1331.1 ± 252.50	1972.32 ± 591.06
	Monolayers	N-cad	1331.1 ± 59.00	1416.54 ± 20.66	681.70 ± 6.28
		Control	918.23 ± 74.48	1031.76 ± 5.38	1346.62 ± 27.60
P1.1MygCAT	Aggregates	N-cad	565.54 ± 61.65	816.14 ± 60.04	—
		Control	498.97 ± 101.95	168.42 ± 15.25	
	Monolayers	N-cad	131.34 ± 3.92	95.70 ± 37.83	—
		Control	238.01 ± 55.44	164.48 ± 58.16	

*Control baby hamster kidney (BHK) cells lacking exogenous N-cadherin (control) and cells stably expressing chicken N-cadherin (N-cad) were transiently transfected with either the p3.7MygCAT or p1.1MygCAT myogenin reporter construct; 24 h later, the cells were harvested and divided in half. One-half of the cells was re-plated as monolayer cultures (monolayers). The other half was cultured as 3-D aggregates in suspension (Aggregates); 60 h later, the cells were collected and extracted, and protein concentration and CAT activity were determined. The average CAT/μg protein for replicate samples (n = 2–4) was calculated, along with the standard deviation (SD). The average CAT activity/μg protein, ±SD, is presented for three independent experiments (Exp 1, 2, 3) done on different days with p3.7MygCAT, and for two independent experiments (Exp 1, 2) with p1.1MygCAT.

layer cultures of control and N-cadherin expressing BHK cells with either the p3.7MygCAT or p1.1MygCAT reporter plasmid; 24 h later, the transiently transfected cells were harvested with trypsin and divided in half. One-half of the cells were subdivided into 2–4 equal portions and the cells placed back in monolayer culture to initiate growth, while the other half was identically subdivided, but the cells placed in 3-D culture to initiate differentiation. After an additional 60 h, the cells were extracted, protein concentration determined, and CAT activity measured, using the diffusion CAT assay as described in Materials and Methods [Martin et al., 1990]. The CAT activity was normalized per μg protein and the average and standard deviation was calculated from the CAT/μg protein values determined for replicate samples (Table I).

We then expressed the CAT activity as a ratio of the average CAT activity/μg protein for cells in 3-D culture (aggregates), divided by the average CAT activity/μg protein for cells in monolayer culture (monolayer). Although the absolute value for CAT activity is dependent on transfection efficiency, the ratio of CAT activity for 3-D versus monolayer culture (aggregate/monolayer) is independent of differences in transfection efficiency. A CAT activity ratio of 1 signifies a lack of change in reporter activity when comparing cells in 3-D versus monolayer culture. On the other hand, a CAT activity ratio of greater than 1 reflects an increase in reporter activity in 3-D versus monolayer cultures. CAT activity ratios calculated from experiments performed on different days were averaged.

We found that the average CAT activity ratio (3-D aggregates/monolayer cells) for control BHK cells without exogenous N-cadherin was approximately 1 (Fig. 5). This result indicates that there is no increase in activity of the myogenin reporter constructs in cadherin-negative control cells stimulated to differentiate by 3-D culture. By contrast, an average CAT activity ratio of approximately 5 and 6.5 was observed for the p3.7MygCAT and p1.1MygCAT reporter constructs, respectively. This result indicates that the activity of both myogenin reporter constructs was increased significantly in N-cadherin-expressing BHK cells stimulated to differentiate by 3-D culture. Moreover, the data suggest that transcription of the endogenous myogenin gene is upregulated, a finding supported by the increase in myogenin protein observed in the N-cadherin-expressing cells in 3-D culture (Fig. 3).

Both the longer p3.7MygCAT and the shorter p1.1MygCAT reporter constructs were tested in BHK cells with or without exogenous N-cadherin, and in 3-D (differentiation) versus monolayer (growth) culture. We consistently found that the activity of p3.7MygCAT was higher than that of p1.1MygCAT (Table I). This

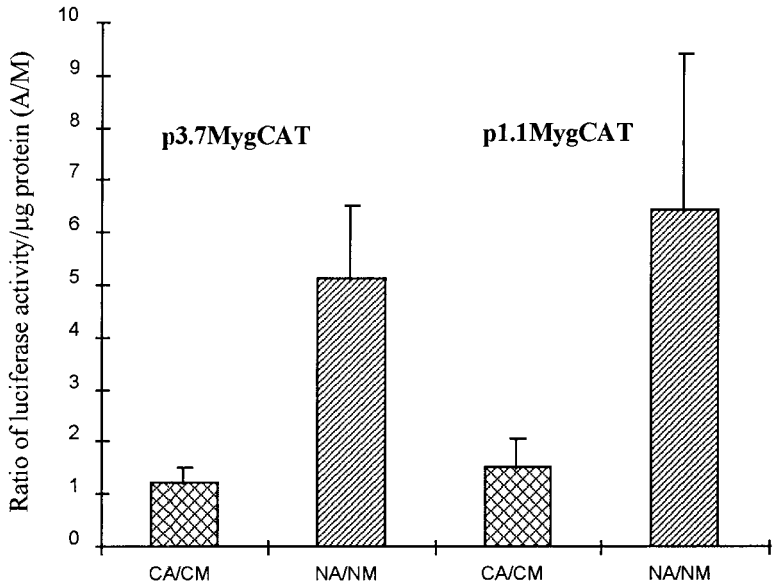

Fig. 5. Ratio of chloramphenicol acetyltransferase (CAT) activity in 3-D versus monolayer culture for baby hamster kidney cells (BHK) cells lacking or expressing exogenous N-cadherin. One 100-mm plate each of 60–80% confluent control BHK cells lacking N-cadherin (C) and BHK cells stably expressing exogenous N-cadherin (N) was transiently transfected, using p3.7MygCAT in three independent experiments, or p1.1MygCAT in two independent experiments as described under Materials and Methods; 72 h later, the cells were harvested with trypsin and divided in half. One-half of the cells were further subdivided equally in 2–4 60-mm dishes as monolayer cultures (M), whereas the other half were subdivided similarly and placed in 3-D culture as aggregates in suspension (**A**). After an additional 60 h, the cells were collected, maintained as replicate samples of equal cell numbers, and then extracted for determination of protein concentration and CAT activity. CAT activity was normalized per μg protein. The average CAT/μg protein and standard deviation were calculated using replicate values (see Table I). The averages for CAT/μg protein were expressed as a ratio for activity in 3-D aggregate (**A**) culture (differentiation condition), divided by activity in monolayer (M) culture (growth condition). The CAT activity ratios presented represent the average and standard deviation of CAT ratios for three independent experiments performed on different days for the p3.7MygCAT construct and two independent experiments for p1.1MygCAT (see Table I). Note that for control (C) cells lacking N-cadherin the average CAT activity ratio is around 1 for both p1.1MygCAT and p3.7MygCAT constructs, indicating no difference in myogenin-CAT activity upon initiation of differentiation by 3-D culture. By contrast, the average CAT activity ratio for cells expressing N-cadherin (N) is approximately 5 and 6.5 for the larger and smaller constructs, respectively. This result indicates that N-cadherin adhesion stimulates CAT activity of both myogenin reporter constructs when the cells are induced to differentiate by placing them in 3-D culture.

difference in activity between these two myogenin reporter constructs is consistent with previous observations by Gibney and Buonanno [1995]. Nevertheless, the average 3-D/monolayer CAT activity ratios were similar for the two myogenin reporter constructs. That is, for both reporter constructs the 3-D/monolayer ratios for CAT activity in control cells lacking N-cadherin was around one, indicating no increase in activity upon initiation of cell differentiation. By contrast, the 3-D/monolayer CAT activity ratios for N-cadherin-expressing cells was approximately 5 for the larger construct and 6.5 for the smaller one. Therefore, our data suggest that both the 3.7 and 1.1 kb upstream regions of the myogenin gene contain *cis*-acting elements responsive to N-cadherin mediated adhesion in 3-D culture.

DISCUSSION

Cadherin-mediated cell-cell adhesion plays multiple roles in the development of striated muscle. It promotes cell recognition of both cardiac and skeletal muscle, induces strong adhesion of cardiac myocytes through intercalated discs, and brings skeletal myoblasts together during their fusion to form a syncytium. Cadherin linkage to the actin cytoskeleton via catenins strengthens cell-cell adhesion and in turn, promotes organization of the actin cytoskeleton, enhancing myofibrillogenesis in cardiomyocytes. In addition, cadherin adhesion

can induce contact inhibition of growth, and alter cell migration of muscle cells [Redfield et al., 1997; Huttenlocher et al., 1998]. In accordance with roles in cell recognition, adhesion, migration, and cytoskeleton organization, perturbation of N-cadherin function in chicken and mouse embryos disturbs muscle morphogenesis, including that of the somites and heart [Radice et al., 1997; Linask et al., 1998; Linask et al., 1997]. The fact that recognizable, albeit abnormal, somites and hearts form at all in embryos with missing or reduced N-cadherin function likely results from expression of additional members of the cadherin family, as well as adhesion proteins from other molecular families, such as NCAM.

Although there is indisputable evidence that cadherin mediated adhesion plays important structural functions, there also is growing evidence it initiates intracellular signals that alter cell growth and differentiation. Our published work with the skeletal myogenic BHK cell line shows that forced expression of N- (or E)-cadherin stimulates muscle differentiation when the cells are induced to differentiate by placing them in 3-D culture. Cadherin-stimulated muscle differentiation does not appear to involve an increase in the level of the skeletal muscle determinant transcription factor, MyoD, or the muscle enhancing transcription factor(s), MEF2. Nor does it appear to involve a change in their phosphorylation. Rather, data presented here show that N-cadherin expression in BHK cells induced to differentiate upregulates the protein level of myogenin, the myogenic transcription factor required for skeletal muscle differentiation. A similar increase in myogenin protein by N-cadherin interactions was observed by Goichberg and Geiger [1998], who exposed mouse skeletal myogenic cells to synthetic beads coated with either recombinant N-cadherin extracellular domain or anti-N-cadherin antibodies. These investigators postulated that surface clustering or immobilization of N-cadherin triggers signaling events that promote myogenic differentiation.

The data presented in this report significantly extend the published work of Goichberg and Geiger [1998] and increase our understanding of how cadherin mediated adhesion stimulates skeletal myogenesis. Our results with myogenin-CAT reporter constructs indicate that N-cadherin adhesion upregulates expression of the myogenin gene in BHK cells induced to differentiate by 3-D culture. An increase in transcription of the endogenous myogenin gene most likely explains the observed increase in myogenin protein stimulated by N-cadherin adhesion in BHK cells induced to differentiate. Thus, our data are consistent with the hypothesis that cadherin adhesion stimulates skeletal myogenesis by upregulating expression of myogenin.

Several questions are raised by our work with the BHK cell line. Why does N-cadherin stimulate differentiation only when the cells are placed in 3-D culture? We speculate that in monolayer culture signals emanating from integrin-mediated cell-matrix adhesion enhance signals initiated by growth factors present in the serum to strongly stimulate cell growth via activation of the MAP kinase pathway [see Aplin et al., 1998]. Together, these pro-proliferative signals probably overwhelm the signal(s) emanating from cadherin adhesion. By contrast, when cells are placed in 3-D culture, cell matrix-adhesion is abolished, reducing the growth stimulating signal. Moreover, cell-cell adhesion is maximized. As a result, the intracellular balance is tipped in favor of signal(s) emanating from cadherin adhesion, which we propose act to coordinately suppress cell growth and stimulate differentiation.

How does cadherin adhesion stimulate transcription of the myogenin gene? Is β-catenin signaling involved? It is unlikely that the cadherin acts exclusively, or perhaps at all, through its ability to upregulate β-catenin and its potential signaling activity, even though expression of exogenous cadherin clearly raises the total level of β-catenin in BHK cells [Redfield et al., 1997] (Fig. 1). Two lines of evidence indicate that increasing β-catenin is not sufficient to stimulate differentiation. First, we previously presented evidence that a mutant N-cadherin capable of increasing β-catenin, but not of inducing cell-cell adhesion, failed to promote BHK differentiation [Redfield et al., 1997]. Second, analysis of the cadherin-responsive 1.1-kb myogenin-CAT reporter construct failed to detect any TCF/LEF binding sites. However, our work does not rule out a role, perhaps even an essential one, for β-catenin in skeletal muscle differentiation. β-Catenin is an essential component of a fully

functional cadherin adhesion system in skeletal muscle cells, although plakoglobin is expressed and may be able to substitute for β-catenin. In addition, it is possible that β-catenin plays a necessary, although not sufficient, signaling role in the commitment or differentiation of the BHK cells, perhaps regulating gene expression even in cells lacking exogenous cadherin.

Although the p1.1MygCAT reporter construct exhibits lower overall CAT activity than displayed by the p3.7MygCAT construct, the activity of both constructs is stimulated similarly by cadherin adhesion in BHK cells induced to differentiate. Thus, it is likely that cadherin-responsive regulatory element(s) lie within the 1.1-kb upstream region of the myogenin gene. The sequence of this region is known, and binding sites for several different transcription factors have been identified [Edmondson et al., 1992]. Two E boxes and a single MEF-2 site have been shown to be important for positive regulation of the myogenin gene in skeletal muscle [Edmondson et al., 1992]. In addition to the E boxes and MEF-2 binding site, there is a CArG binding site for serum response factor (SRF), an NF-1 site, and possible Sp1 binding sites. Future studies will define more closely the promoter region of myogenin responsive to cadherin adhesion.

ACKNOWLEDGMENTS

The monoclonal antibody F5D developed by Dr. W.E. Wright was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa. The authors are grateful to Dr. Alejandro Peralta Soler for discussions, for reading the manuscript, and for helping in the preparation of the figures.

REFERENCES

Andrés V, Walsh K. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol 132:657–666.

Aplin AE, Howe A, Alahari SK, Juliano RL. 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin cell adhesion molecules, and selectins. Pharmacol Rev 50:197–263.

Arnold H-H, Braun T. 1996. Target inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review. Int J Dev Biol 40:345–363.

Auradé F, Pinset C, Chafey P, Gros F, Montarras D. 1994. Myf5, MyoD, myogenin and MRF4 myogenic derivatives of the embryonic mesenchymal cell line C3H10T1/2 exhibit the same adult muscle phenotype. Differentiation 55:185–192.

Bader D, Masaki T, Fischman DA. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J Cell Biol 95:763–770.

Behrens J, von Kries, JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W. 1996. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 382:638–642.

Bienz M. 1998. TCF: transcriptional activator or repressor. Curr Opin Cell Biol 10:366-372.

Brand-Saberi B, Gamel AJ, Krenn V, Miller TS, Wilting J, Christ B. 1996. N-cadherin is involved in myoblast migration and muscle differentiation in the avian limb bud. Dev Biol 178:160–173.

Buckingham M. 1995. Skeletal muscle development and the role of myogenic regulatory factors. Biochem Soc Trans 24:506–509.

Buonanno A, Edmondson DG, Hayes WP. 1993. Upstream sequences of the *myogenin* gene convey responsiveness to skeletal muscle denervation in transgenic mice. Nucleic Acids Res 21:5684–5693.

Cadigan KM, Nusse R. 1997. Wnt signaling: a common theme in animal development. Genes Dev 11:3286–3305.

Choi J, Costa ML, Mermelstein CS, Chagas C, Holtzer S, Holtzer H. 1990. MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. Proc Natl Acad Sci USA 87:7988–7992.

Cossu G, Tajbakhsh S, Buckingham M. 1996. How is myogenesis initiated in the embryo. Trends Gene Ther 12: 218–223.

Daniel JM, Reynolds AB 1997. Tyrosine phosphorylation and cadherin/catenin function. BioEssays 19:883–891.

Davis RL, Weintraub H, Lassar AB. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000.

Donalies M, Cramer M, Ringwald M, Starzinski-Powitx A. 1991. Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. Proc Natl Acad Sci USA 88:8024–8028.

Duband J-L, Volberg T, Sabanay I, Thiery JP, Geiger B. 1988. Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. Development 103:325–344.

Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN. 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF2. Mol Cell Biol 12:3665–3677.

Emerson CP. 1993. Skeletal myogenesis: genetics and embryology to the fore. Curr Opin Genet Dev 3:265–274

Fagotto F, Gumbiner BM. 1996. Cell contact-dependent signaling. Dev Biol 180:445–454.

Fredette BJ, Ranscht B. 1994. T-cadherin expression delineates specific regions of the developing motor axon-hindlimb projection pathway. J Neurosci 14:7331–7346.

George-Weinstein MJ, Gerhart J, Blitz J, Simak E, Knudsen KA. 1997. N-cadherin promotes the commitment and differentiation of skeletal muscle precursor cells. Dev Biol 185:14–24.

Gibney G, Buonanno A. 1995. Analysis of neural-responsive myogenin upstream sequences by myoblast implantation. Dev Biol 172:614–624.

Goichberg P, Geiger B. 1998. Direct Involvement of N-cadherin-mediated signaling in muscle differentiation. Mol Biol Cell 9:3119–3131.

Grunwald GB. 1993. The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. Curr Opin Cell Biol 5:797–805.

Grunwald GB. 1996. Discovery and analysis of the classical cadherins. Adv Mol Cell Biol 16:63–112.

Gumbiner BM. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84:345–357.

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

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

Holt CE, Lemaire P, Gurdon JB. 1994. Cadherin-mediated cell interactions are necessary for the activation of MyoD in *Xenopus* mesoderm. Proc Natl Acad Sci USA 91: 10844–10848.

Huttenlocher A, Lakonishok M, Kinder M, Wu S, Truong T, Knudsen KA, Horwitz AF. 1998. Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. J Cell Biol 141:515–526.

Imanaka-Yoshida K, Knudsen KA, Linask KK. 1998. N-cadherin is required for the differentiation and initial myofibrollogenesis of chick cardiomyocytes. Cell Motil Cytoskeleton 39:52–62.

Inuzuka H, Redies C, Takeichi M. 1991. Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development. Development 113:959–967.

Johnson KR, Lewis JE, Li D, Wahl A, Peralta Soler A, Knudsen KA, Wheelock MJ. 1993. P- and E-cadherin are in separate complexes in cells expressing both cadherins. Exp Cell Res 207:252–260.

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

Knudsen KA, Horwitz AF. 1994. The plasma membrane of the muscle fiber: Adhesion molecules. In: Engel AG, Franzini-Armstrong, C, editors. Myology: basic and clinical. 2nd Ed. New York: McGraw-Hill. p 223–241.

Knudsen KA, Myers L, McElwee SA. 1990. A role for a Ca^{2+}-dependent adhesion molecule, N-cadherin in myoblast interaction during myogenesis. Exp Cell Res 188: 175–184.

Knudsen KA, Peralta Soler A, Johnson KR, Wheelock MJ. 1995. Interaction of α-actinin with the cadherin/catenin cell-cell adhesion complex via α-catenin. J Cell Biol 130: 67–77.

Knudsen KA, Frankowski C, Johnson KR, Wheelock MJ. 1998. A role for cadherins in cellular signaling and differentiation. J Cell Biochem Suppl 30/31:168–176.

Kühl M, Wedlich D. 1997. Wnt signalling goes nuclear. BioEssays 19:101–104.

Lassar A, Münsterberg A. 1994. Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. Curr Opin Cell Biol 6:432–442.

Linask KK, Knudsen KA, Gui YH. 1997. N-cadherin-catenin interaction: necessary component of cardiac cell compartmentalization during early vertebrate heart development. Dev Biol 185:148–164.

Linask KK, Ludwig C, Han J-D, Liu X, Radice GL, Knudsen KA. 1998. N-cadherin/catenin-mediated morphoregulation of somite formation. Dev Biol 202:85–103.

Ludolph DC, Konieczy SF. 1995 Transcription factor families: muscling in on the myogenic program. FASEB J 9:1595–1604.

Martin JD. 1990. Application of the two-phase partition assay for chloramphenicol acetyltransferase (CAT) to transfections with simian virus 40-CAT plasmids. Anal Biochem 191:242–246.

McDonald KA, Horwitz AF, Knudsen KA. 1995. Adhesion molecules and skeletal myogenesis. Semin Dev Biol 6:105–116.

Mege RM, Goudou D, Diaz C, Nicoloet M, Garcia L, Geraud G, Gieger F. 1992. N-cadherin and N-CAM in myoblast fusion: compared localization and effect of blockade by peptides and antibodies. J Cell Sci 103:897–906.

Megeney LA, Rudnicki MA. 1995. Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol 73:723–732.

Miller JR, Moon RT. 1996. Signal transduction through β-catenin and specification of cell fate during embryogenesis. Genes Dev 10:2527–2539.

Olsen EN. 1992. Interplay between proliferation and differentiation within the myogenic lineage. Dev Biol 154: 261–272.

Olson E, Arnold H, Rigby P, Wold B. 1996 Know your neighbors: three phenotypes in null mutant of the myogenic bHLH gene mrf4. Cell 85:1–4.

Orsulic S, Peifer M. 1996. Cell-cell signalling: wingless lands at last. Curr Biol 6:1363–1367.

Peifer M. 1995. Cell adhesion and signal transduction: the Armadillo connection. Trends Cell Biol 5:224–229.

Peralta Soler A, Knudsen KA. 1994. N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. Dev Biol 162:9-17.

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

Redfield A, Nieman MT, Knudsen KA. 1997. Cadherins promote skeletal muscle differentiation in three-dimensional cultures. J Cell Biol 138:1323–1331.

Rosenberg P, Esni F, Sjödin A, Larue L, Carlsson L, Gullberg D, Takeichi M, Kemler R, Semb H. 1997. A potential role of R-cadherin in striated muscle formation. Dev Biol 187:55–70.

Rudnicki MA, Jaenisch R. 1995. The MyoD family of transcription factors and skeletal myogenesis. BioEssays 17: 203–209.

Skerjanc IS, Slack RS, McBurney MW. 1994 Cellular aggregation enhances MyoD-directed skeletal myogenesis in embryonal carcinoma cells. Mol Cell Biol 14:8451–8459.

Takeichi M. 1990. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 59:237–252.

Takeichi M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Sci USA 251:1451–1455.

Tonks NK, Neel BG. 1996. From form to function: signaling by protein tyrosine phosphatases. Cell 87:365–368.

Tsukita S, Tsukita S, Nagafuchi A, Yonemura S. 1992. Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. Curr Opin Cell Biol 4:834–839.

Weintraub H. 1993. The MyoD family and myogenesis: redundancy, networks and thresholds. Cell 75:1241–1244.

Wheelock MJ, Knudsen KA, Johnson KR. 1996. Membrane-cytoskeleton interactions with cadherin cell adhesion proteins: roles of catenins as linker proteins. Curr Top Membr 43:169–185.

Wright WE, Binder M, Funk W. 1991. Cyclic amplification and selection of targets (CASTing) for the myogenin consensus binding site. Mol Cell Biol 11:4101–4110.

Yap AS, Brieher, WM, Gumbiner BM. 1997. Molecular and functional analysis of cadherin-based adherens junctions. Annu Rev Cell Dev Biol 13:119–146.

Yun KS, Wold B. 1996. Skeletal muscle determination and differentiation: Story of a core regulatory network and its context. Curr Opin Cell Biol 8:877–889.

Zeschnigk M, Kozian D, Kuch C, Schmoll M, Starzinski-Powitz A. 1995. Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. J Cell Sci 108: 2973–2981.