Recruitment of β-catenin to cadherin-mediated intercellular adhesions is involved in myogenic induction

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SUMMARY

Cadherin-mediated cell adhesion is involved in muscle differentiation from early stages of myogenic induction to late stages of myoblast interaction and fusion. β-Catenin is a major constituent of cadherin-based adherens junctions and also serves as a signal transduction molecule that regulates gene expression during development. In this study, we explored the involvement of β-catenin in myogenic differentiation. We show here that shortly after a switch from growth to differentiation medium, β-catenin translocates to cell-cell junctions and its levels increase. We further show that elevation of β-catenin levels, induced either by inhibition of its breakdown, using LiCl, or by its overexpression, suppresses the formation of adherens junctions, resulting in a sharp decline in myogenin expression and an arrest of myogenic progression. Recruitment of β-catenin to adherens junctions after transfection with N-cadherin restores myogenin expression in the transfected cells. These results suggest that increased cadherin-mediated adhesion and translocation of β-catenin to adherens junctions are involved in activating the early steps of myogenic differentiation.

Key words: β-Catenin, Myogenesis, Cell adhesion, Myogenin

INTRODUCTION

β-Catenin plays a central role in regulating cell fate, due to its participation in the assembly of adherens junctions and its ability to directly activate the transcription of specific target genes (for recent reviews see Ben-Ze'ev, 1997; Ben-Ze'ev and Geiger, 1998; Bullions and Levine, 1998; Willert and Nusse, 1998). At cell-cell junctions, β-catenin links cadherin molecules via their intracellular domains to the actin-based cytoskeleton (Ben-Ze'ev, 1997; Geiger et al., 1995). A pivotal step in Wnt/Wingless (Wg) signaling, occurs when β-catenin binds to transcription factors of the LEF-1/TCF family, translocates to the nucleus and activates the expression of specific genes (Porfiri et al., 1997; Gumbiner, 1998; He et al., 1998; Hsu et al., 1998; Simcha et al., 1998; Wodarz and Nusse, 1998; Mann et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999). In addition, β-catenin-dependent transactivation may also involve a CREB-site-dependent mechanism, as was recently suggested for Wnt-induced WISP-1 activation (Xu et al., 2000). Further interest in β-catenin-activated target gene expression was raised by the finding that Wnt signaling is involved in embryonic morphogenesis (for review see Wodarz and Nusse, 1998) and that accumulation of β-catenin, due to mutations in the adenomatous polyposis coli (APC) gene, or in the β-catenin gene itself, are associated with colon cancer and other malignancies (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Among the genes whose expression was found to be regulated by β-catenin are c-myc (He et al., 1998), cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999), c-jun (Mann et al., 1999) and PPARδ (He et al., 1999). Recent studies have also indicated that the interactions of β-catenin with cadherin and LEF-1 might be antagonistic. Thus, activation of a LEF-1-responsive reporter in cells expressing high levels of β-catenin (such as the SW480 colon carcinoma cells) and nuclear localization of β-catenin could be inhibited by over-expressing N-cadherin or α-catenin in these cells (Sadot et al., 1998; Simcha et al., 1998). Moreover, overexpression of the soluble, intracellular domain of N- or E-cadherin inhibits β-catenin-dependent transactivation and blocks its degradation (Sadot et al., 1998; Orsulic et al., 1999).

Several lines of evidence suggest that cadherin-mediated interactions are also involved in the regulation of skeletal myogenesis (Knudsen et al., 1990; Mege et al., 1992; George-Weinstein et al., 1997; Redfield et al., 1997; Goichberg and Geiger, 1998; Linask et al., 1998; Seghatoleslami et al., 2000). In a previous study, we demonstrated that stimulation of cadherin-mediated adhesion in proliferating myoblasts, by cadherin-reactive beads (coated with anti-N-cadherin antibodies or with the extracellular domain of N-cadherin), facilitates the expression of the muscle regulatory protein myogenin, and induces other myogenic events, including growth arrest and myotube formation (Goichberg and Geiger, 1998). In addition, shortly after incubation with N-cadherin-reactive beads, myoblasts aggregate into coherent arrays, develop adherens junctions and recruit β-catenin to cell-cell contact sites (Goichberg and Geiger, 1998; Levenberg

et al., 1998b). This is consistent with immunohistochemical analysis of β-catenin expression in avian embryos, which indicated that its association with intercellular junctions is correlated with somitogenesis (Linask et al., 1998).

In this study, we examined the role of β-catenin in the regulation of muscle differentiation. We report here on major changes in both the level and intracellular distribution of β-catenin in differentiating myoblasts. These changes are accompanied by an increase in adherens junction formation, followed by an increase in β-catenin level and in myogenin expression. Surprisingly, overexpression of β-catenin was found to reduce intercellular adhesion and inhibit myogenin expression. Our results suggest that cadherin-mediated adhesion and translocation of β-catenin to adherens junctions are involved in the promotion of myogenesis via activation of myogenin expression.

MATERIALS AND METHODS

Cell culture

The mouse and rat skeletal myoblast cell lines C2 and L8 (Yaffe and Saxel, 1977a; Yaffe and Saxel, 1977b), were kindly provided by Dr D. Yaffe (The Weizmann Institute of Science, Rehovot). The cells were maintained in either growth medium (FS) or differentiation medium (DM), plated at subconfluent densities and incubated at 37°C in a humidified atmosphere containing 8% CO_2. C2 cells were cultured in DME medium supplemented with 20% heat-inactivated fetal calf serum (FCS; BioLabs Ltd, Israel), glutamine and antibiotics. L8 cells were cultured in Waymouth medium containing 15% FCS. To trigger differentiation, C2 myoblasts were either plated at high density (approximately 80% confluence), or stimulated by DM, containing insulin (Humulin R, Lilly France S.A., France; 4 IU/100 ml) and 10% horse serum in DMEM. Myogenic differentiation of L8 cells was induced by changing the growth medium to DMEM containing 2% heat-inactivated horse serum (Biological Industries, Israel) and 4 IU/100 ml insulin.

Treatment with LiCl

C2 cells were plated on culture dishes or coverslips, and incubated with medium containing 30 mM of either LiCl or NaCl (as control) for the indicated periods of time. The cells were fixed and subjected to immunocytochemical staining for β-catenin and myogenin.

Immunoblot analysis

Cells were washed with PBS and lysed with sample buffer (Laemmli, 1970). Protein concentration was determined using the Bradford Reagent (Bio-Rad Laboratories, CA, USA) and equal amounts of protein (20-50 μg) were loaded on the gel. To separate between Triton X-100-soluble and -insoluble proteins, cells were extracted directly from culture dishes for 2 minutes in 50 mM MES (2-[*N*-morpholino] ethanesulfonic acid) buffer, pH 6.0, containing 0.5% Triton X-100, 2.5 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 0.1 mM aprotinin, leupeptin, pervanadate, NaF and NaPPi. The Triton-insoluble fraction was scraped off the plate with a rubber policeman in the same volume of the above buffer. Identical aliquots of both fractions were boiled in Laemmli's sample buffer and subjected to 9% SDS-polyacrylamide gel electrophoresis followed by electroblotting onto Hybond-C nitrocellulose membranes (Amersham Co., UK). The membranes were blocked for 1 hour with a 4% solution of dry milk in PBS, and incubated overnight at 4°C with the primary antibodies, diluted in PBS or TBS. After washing in PBS, the membranes were incubated for 45 minutes at room temperature with HRP-conjugated goat anti-mouse or anti-rabbit IgG (Amersham Co.), and immuno-reactive bands were visualized using the Enhanced ChemiLuminiscence System (ECL, Amersham).

Immunochemical reagents and procedures

C2 and L8 myoblasts, cultured on glass coverslips, were washed with PBS, fixed and permeabilized for 4 minutes at room temperature with 0.5% Triton X-100, 3% paraformaldehyde in PBS, and post-fixed for an additional 25 minutes with 3% paraformaldehyde. The antibodies used in this study were: monoclonal anti-pan-cadherin (CH19), monoclonal anti-chicken N-cadherin (ID7.2.3), monoclonal anti-β-actin, rabbit anti-β-catenin, rabbit anti-pan-cadherin and rabbit anti-β-catenin (all purchased from Sigma Chemical Co., Israel); monoclonal anti-plakoglobin (γ-catenin) was purchased from Transduction Laboratories, Lexington, KY, USA; rabbit anti-HA antibody was obtained from Dr M. Oren (Weizmann Institute of Science, Rehovot). Anti-myogenin antibody was provided by Drs B. Winter and H. H. Arnold (Technical University, Braunschweig, Germany), and monoclonal anti-MyoD antibody was purchased from Novocastra Laboratories, Ltd, Newcastle upon Tyne, UK. The secondary antibodies used were Cy3-conjugated goat anti-mouse IgG (Jackson Immuno-Research Labs, Inc. West-Groove, PA, USA) and Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Oregon, USA). Nuclei were stained for 10 minutes with 2.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole; Sigma Chemical Co.), and the cells were mounted in Elvanol (Mowiol 4-88, Hoechst, Frankfurt, Germany). Immunofluorescent images were visualized with either an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped for multiple fluorescence examination, or by the DeltaVision system (Applied Precision Inc., Issaqua, WA, USA), as indicated.

Plasmids and transient transfections

The preparation of a vector expressing β-catenin containing a Ser(33)-to-Tyr mutation (β-cateninY33) was previously described (Damalas et al., 1999). This mutant lacks one of the major GSK3-phosphorylation sites and is thus relatively resistant to proteasomal degradation (Aberle et al., 1997; Orford et al., 1997). The full length chicken N-cadherin cloned into the pECE expression vector and the N-cadherin tail-GFP fusion protein were described elsewhere (Levenberg et al., 1998a; Sadot et al., 1998). A construct expressing HA-tagged skeletal tropomyosin α was obtained from Dr D. Helfman (Cold Spring Harbor Laboratory, NY, USA).

C2 cells were plated on coverslips at a density of 2×10^5 cells per 35 mm dish. When approaching confluence, the cells were transfected with 4 μg DNA (2 μg of expression vector and 2 μg of empty vector) using 8 μl Lipofectamine and 6 μl Plus reagent (GibcoBRL, Life Technologies, UK). After 3 hours of incubation with the transfection mixture in serum-free medium, the cells were treated for an additional 48 hours with DM. The cells were then fixed and double immunolabeled, as described above.

Transactivation assays

C2 cells were plated at a density of 2×10^5 cells per 35 mm dish, and 18 hours later co-transfected with 1 μg of the LEF-1 reporter, TOPFLASH (van de Wetering et al., 1997), 0.5 μg of LEF-1 and 1 μg of β-cateninY33, either alone or together with 1 μg of N-cadherin tail or the full length chicken N-cadherin. pCDNA3 coding for β-galactosidase (0.5 μg) was used to normalize transfection efficiency. Luciferase and β-galactosidase activities were determined 48 hours after transfection and normalized as described (Sadot et al., 1998).

Retroviral infection and production of pools of stably infected cells

Preparation of the retroviral vector DNA expressing β-cateninY33 and containing the HA tag was described (Damalas et al., 1999). Puromycin-resistant pools of C2 cells infected with the above DNA, or with pBabe-puro retroviral DNA, as control, were produced as described (Damalas et al., 1999). Briefly, retroviral stocks were

obtained by transfecting each of the constructs into 293T cells, by the calcium-phosphate co-precipitation method, together with the ψ^- ectopic packaging vector, pSV-ψ^-E-MLV, providing the packaging helper function. Virus-containing supernatants were collected 24-72 hours post-transfection, at 6 hour intervals, pooled together, filtered and added in the presence of polybrene (Sigma Chem. Co.) to the C2 myoblasts, plated 2×10^5 cells per 6 cm dish. The supernatants were added 3 times at 4 hour intervals. After the last infection, the cells were incubated with fresh growth medium (FS) for 18 hours, and then plated into the selective medium (FS supplemented with 2 μg/ml puromycin; Sigma Chem. Co.). The medium was changed every two days, and pools of stably infected cells were collected after 2 weeks.

RESULTS

Changes in cell-cell contacts and catenin levels during myogenic differentiation

To monitor the assembly of adherens junctions during the myogenic process, we examined the changes in the distribution of α-, β-, and γ-catenin (plakoglobin), at different stages of muscle differentiation in culture. Immunofluorescence labeling demonstrated that C2 and L8 myoblasts adhere poorly to each other and that the three catenins are not enriched along cell-cell interfaces (Fig. 1A-D). α-catenin and plakoglobin exhibited diffuse cytoplasmic distribution, while β-catenin was also enriched in the nucleus (Fig. 1B,D, arrowheads). Upon the onset of differentiation (namely when myocytes stop proliferating and start to express muscle-specific proteins), an association of the three catenins with cell-cell contacts became apparent (Fig. 1E-H, arrows). When myotubes formed, adherens junctions were no longer detected and the staining for cadherin (not shown) and catenins was mostly diffuse (Fig. 1I-L). Immunoblot analysis revealed a major increase (approximately 6-fold) in the level of cadherin and a more moderate increase in β-catenin and plakoglobin levels in differentiating muscle cells, which was apparent within 8 hours after medium switch (Fig. 2B and C). A similar increase in cadherin and β-catenin levels was observed in differentiating L8 cells, however the changes were apparent 24 to 48 hours later than in C2 cells (data not shown). This is in agreement with the slower rate of differentiation characteristic to L8 cells (Yaffe and Saxel, 1977a).

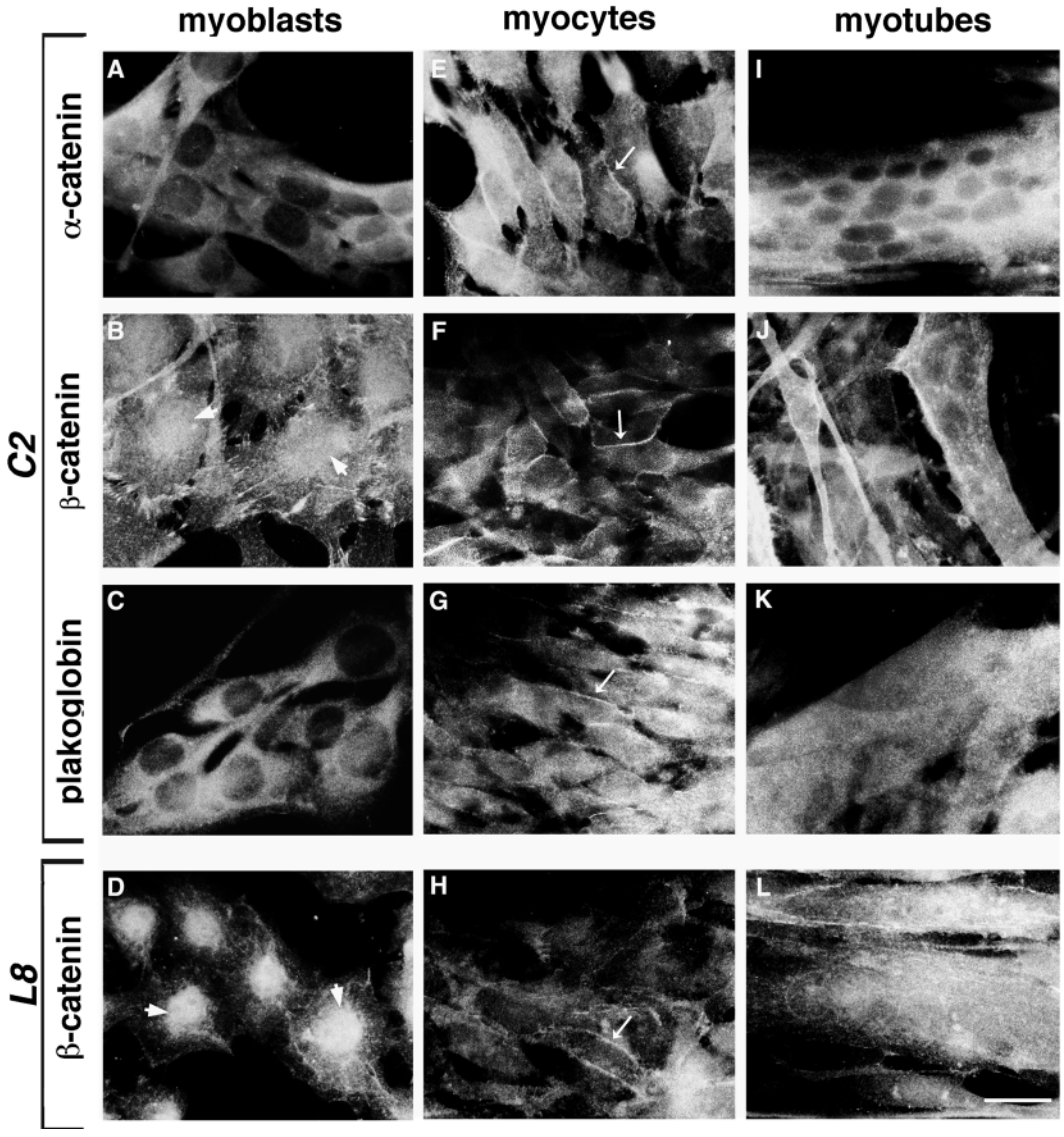

Fig. 1. Changes in the subcellular localization of catenins during differentiation of C2 and L8 myogenic cell lines. C2 and L8 cells were fixed and immunolabeled either for α-catenin, β-catenin or plakoglobin at three distinct stages of myogenesis: (i) Subconfluent proliferating cells, 18 hours after plating, that were defined as myoblasts (A-D); (ii) cells approaching confluence, cultured in differentiation medium (DM) for 24-48 hours that express myogenin as well as some muscle-specific structural proteins, are defined as myocytes (E-H); (iii) fused cells, after incubation for 48-96 hours in DM, displaying elongated morphology, multinucleation, and expressing a full repertoire of muscle-specific proteins, that were defined as myotubes (I-L). Note that β-catenin is present in the nuclei of myoblasts (arrowheads, B,D), while in myocytes β-catenin is mostly localized at cell-cell contact sites (arrows, in F and H). Plakoglobin and α-catenin have diffuse cytoplasmic distribution in myoblasts and are present at the cell-cell contacts in myocytes (arrows, E and G). Bar, 20 μm.

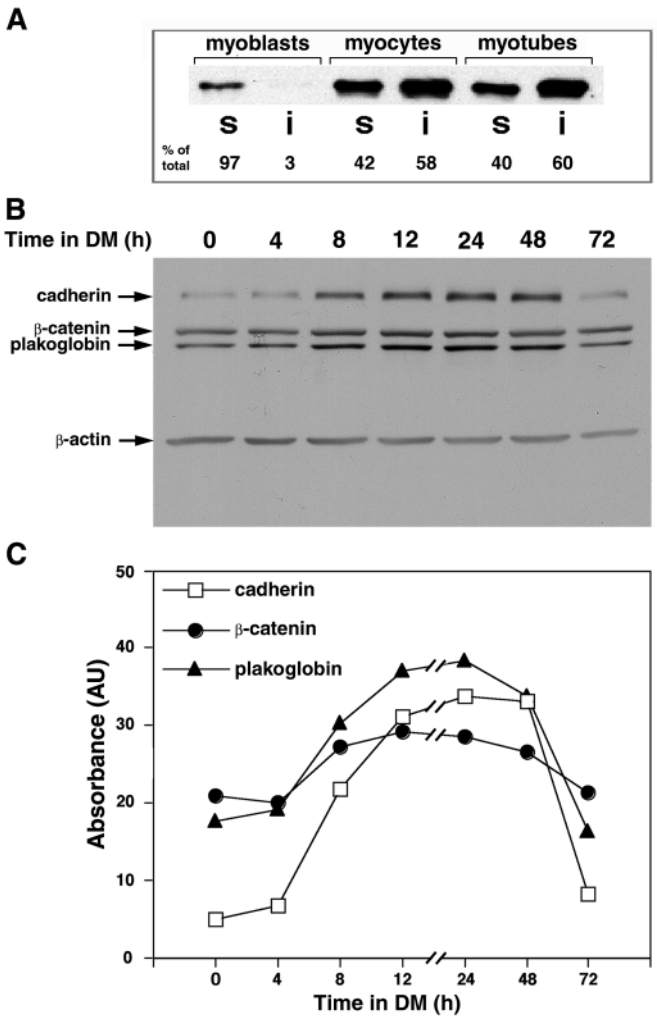

Fig. 2. Changes in the level of adherens junction components during muscle cell differentiation. Cultured C2 cells were harvested at the indicated time points following induction with differentiation medium (DM). (A) Comparison of the Triton-soluble (s) and insoluble (i) fractions of cultured C2 cells at the myoblast, myocyte and myotube stages. Cell extracts were subjected to immunoblot analysis with anti-β-catenin antibody. Note that while nearly all β-catenin in myoblasts was associated with the Triton-soluble fraction, in differentiating cells (myocytes or myotubes) about 60% of β-catenin was associated with the Triton-insoluble fraction. (B) Equal amounts of protein were subjected to sequential western blot analysis with anti-cadherin, anti-β-catenin, anti-plakoglobin, and anti-β-actin antibodies. Note the increase in the levels of both cadherin and catenins following incubation with DM and the characteristic decrease in β-actin levels. (C) The relative levels of cadherin and the two catenins as determined by densitometric measurements.

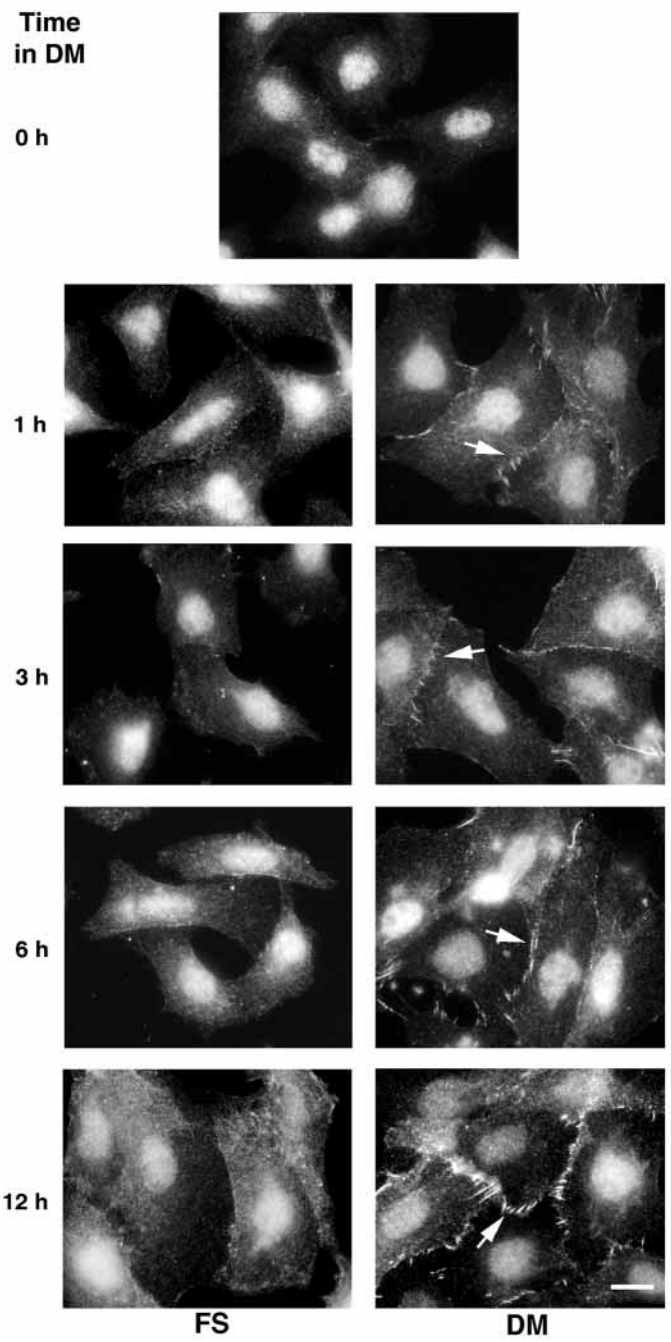

Fig. 3. Induction of cell-cell contacts in L8 myoblasts treated with differentiation medium. L8 cells were plated for 18 hours in growth medium (FS) and then incubated in differentiation medium (DM) for the indicated time periods. Following fixation and permeabilization, the cells were immunolabeled for β-catenin and digital images were acquired. Arrows point to cell-cell contacts. Note the increase in the prominence and intensity of junctional β-catenin after incubation with DM (arrows). Bar, 10 μm.

Detergent extraction of differentiating cells followed by western blot analysis indicated that while in myoblasts, nearly all β-catenin was Triton X-100-soluble, in differentiated cells about 60% of β-catenin was associated with the Triton X-100-insoluble fraction (Fig. 2A), suggesting that it became associated with the cytoskeleton. The levels of β-catenin in myoblasts appear to depend on the plating density of cells. Thus, in sparse cells the total amount of β-catenin protein was much lower than in densely plated myoblasts. Therefore, the extent of increase in β-catenin level in differentiating myogenic cells varied between sparse cell populations (Fig. 2A) and densely plated cells (Fig. 2B,C).

Junctional localization of β-catenin is induced shortly after removal of the growth medium

To test the kinetics of adherens junction formation following

the induction of differentiation (i.e. replacement of FS with DM), L8 cells which have low levels of junctional β-catenin in the non-differentiated state, were incubated for different time periods with DM, and then fixed and stained for β-catenin. As shown in Fig. 3, within 1 hour after medium switch, β-catenin was already associated with cell-cell junctions in L8 myoblasts (Fig. 3, arrows) and its presence in adherens junctions became more prominent upon longer incubation with DM. A similar increase in junctional β-catenin was detected shortly after growth factor deprivation of C2 myoblasts (data not shown).

The effect of increased levels of β-catenin on myogenin expression

In view of the apparent correlation between the increase in β-catenin levels and the arrest of myogenesis, we determined whether artificial elevation of β-catenin can induce myogenin expression. This was carried out by lithium-induced stabilization of β-catenin, as well as by its forced overexpression.

Li^+ ions were shown to inhibit GSK3β activity, resulting in the elevation of β-catenin protein levels (Stambolic et al., 1996; Aberle et al., 1997; Orford et al., 1997; Salomon et al., 1997). In addition, Linask et al. demonstrated that LiCl-treated chicken embryos display anomalous somites (Linask et al., 1998), similar to those obtained after treatment with N-cadherin-perturbing antibodies.

To examine the effect of excess β-catenin on muscle differentiation, we treated myoblasts with 30 mM LiCl. This treatment inhibited the fusion of both C2 and L8 cells into myotubes when induced to differentiate as above (data not shown). The removal of LiCl after several days of treatment, resulted in a rapid progression of myogenesis (formation of myotubes after 24 hours of stimulation), indicating that prolonged LiCl treatment was not toxic to these cells and did

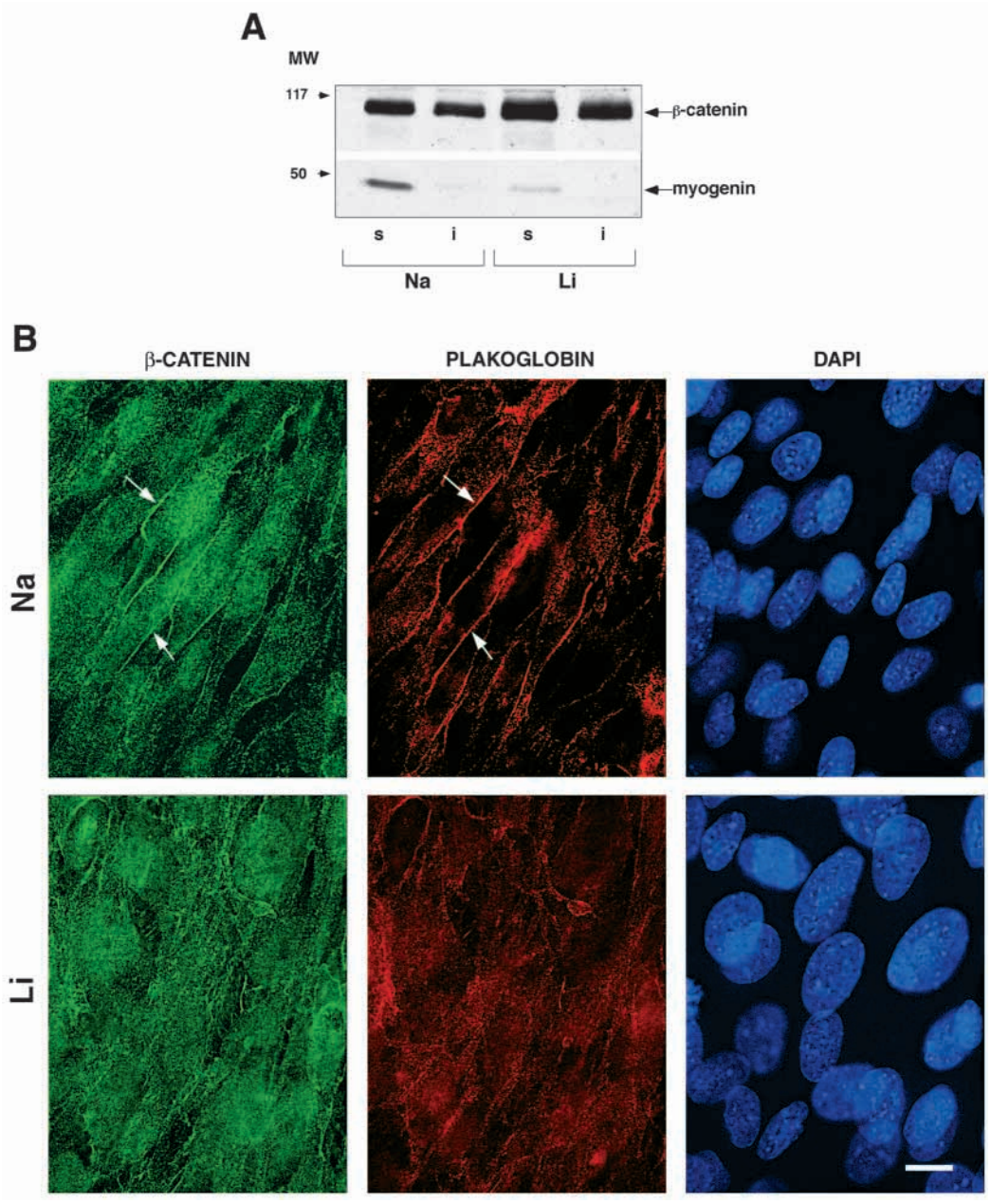

Fig. 4. LiCl treatment inhibits myogenin expression and adherens junction formation in cultured C2 myocytes. (A) Sequential immunoblot analysis for β-catenin and myogenin of the Triton-soluble (s) and insoluble (i) fractions of differentiating C2 myocytes. The cells were stimulated with DM for 30 hours, and then incubated for an additional period of 16 hours in the presence of either 30 mM NaCl (Na) or 30 mM LiCl (Li). Note the elevation in β-catenin levels and the suppression of myogenin levels following LiCl treatment. (B) Double immunolabeling of adherens junctions of C2 cells for β-catenin and plakoglobin after NaCl or LiCl treatment. C2 cells were cultured till confluence and treated as in A above. The cells were double stained with anti-β-catenin and anti-plakoglobin antibodies. Nuclei were visualized by DAPI staining. Digital images were acquired. Arrows point to identical positions along cell-cell contacts in NaCl treated cells. Note the apparent decrease in adherens junctions induced by LiCl, which correlates with the decrease in myogenin levels. Bar, 10 μm.

not irreversibly suppress their myogenic capacity. Moreover, western blot analysis showed that the addition of LiCl to myocytes, which already expressed myogenin, results in an overall reduction in myogenin expression (Fig. 4A). On the other hand, when LiCl was added to terminally differentiated myotubes, it had no apparent effect on cell morphology and did not affect myogenin expression (data not shown), indicating that increased levels of β-catenin specifically affected the activity and/or expression of relatively early muscle-regulatory factors. Immunofluorescent labeling of LiCl-treated cells for plakoglobin and β-catenin (Fig. 4B), as well as cadherins (not shown), revealed a major disruption of adherens junctions. This suggests that either the destruction of cell-cell junctions, or accumulation of β-catenin, inhibited myogenin expression and blocked myogenesis.

To further explore the specific role of β-catenin in this process, we examined retroviral infected C2 cells, stably overexpressing β-catenin (Fig. 5A,B). Interestingly, while the increase in β-catenin levels was modest (Fig. 5B) (~20% over the control), the β-catenin overexpressing cells did not express myogenin even after stimulation with differentiation medium for 72 hours (Fig. 5C). However, overexpression of β-catenin did not affect the basal level of MyoD expression when compared to control cells transfected with the puromycin gene only (Fig. 5C, time 0). Subsequently, MyoD protein levels decreased in cells overexpressing β-catenin (Fig. 5C, 24-72 hours). This late decline could be attributed to the fact that MyoD expression is maintained in cultured C2 cells both by autoregulatory stimulation and by a feedback loop from downstream muscle specific transcription factors, such as myogenin (for review see Yun and Wold, 1996), which is impaired in these cells. It appears that the effect of β-catenin on myogenic differentiation is not highly dependent on LEF-mediated transcription, since the activity of the LEF-reporter (TOPFLASH) is only slightly elevated in C2 cells overexpressing β-catenin (Fig. 5D, and see below).

To directly examine whether the inhibitory effect of β-catenin on myogenesis is attributable to its transactivation activity or to its effect on adherens junction integrity, C2 cells were co-transfected with the mutant β-cateninY33 that is less sensitive to degradation, and either full length N-cadherin or the cytoplasmic domain of N-cadherin (N-cadherin tail). Both molecules were shown to stabilize β-catenin against proteasomal degradation (Sadot et al., 1998). As demonstrated in Fig. 6A, β-cateninY33 inhibited myogenin expression and this inhibition was fully reversed by N-cadherin, but not by its cytoplasmic tail. Interestingly, β-cateninY33 had no apparent effect on MyoD expression (Fig. 6B).

Overexpression of the N-cadherin tail inhibited myogenin expression in the transfectants, while expression of skeletal muscle tropomyosin α had no such effect (data not shown). In contrast, the expression of MyoD was not significantly affected by transfection with the N-cadherin tail (Fig. 6B). Examination of the effect of N-cadherin, or its cytoplasmic tail, on β-catenin-mediated transactivation indicated that both molecules inhibited TOPFLASH-reporter luciferase activity (Fig. 6C). While the N-cadherin tail was much more effective than the full-length molecule in suppressing β-catenin-driven transactivation (Fig. 6C), it did not affect the expression of myogenin in β-catenin overexpressing cells (Fig. 6A). Intact N-cadherin, on the other hand, fully restored myogenic differentiation in these cells (Fig. 6A). As indicated in Fig. 6A, overexpression of functional N-cadherin results in higher

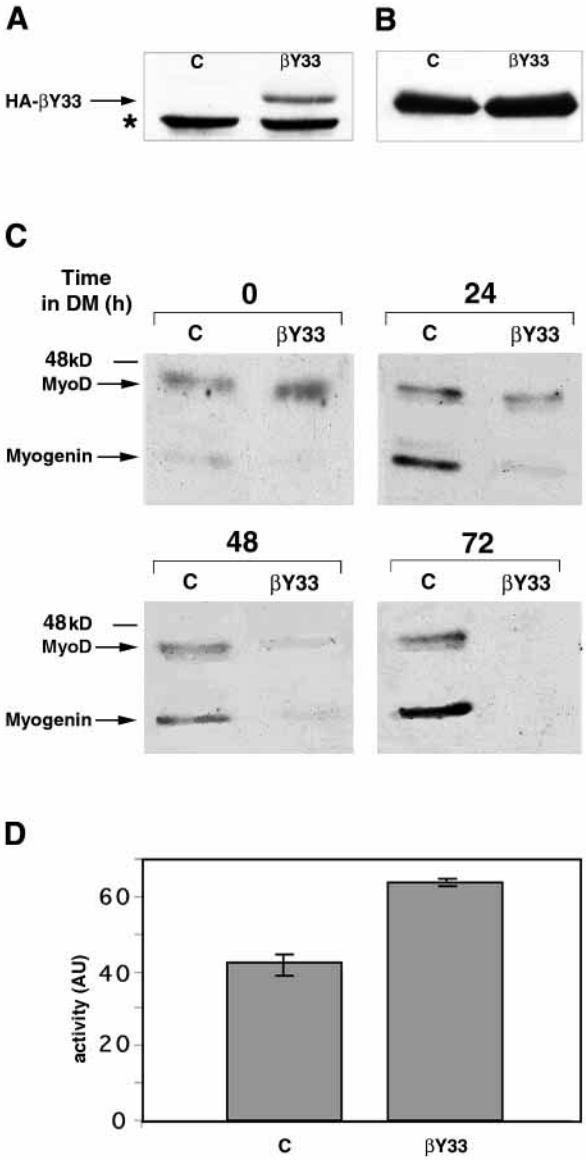

Fig. 5. Stable overexpression of β-catenin inhibits myogenesis in C2 cells. C2 myoblasts were infected with a recombinant retrovirus encoding either HA-tagged β-cateninY33 (βY33) or the puromycin resistance vector (c), as described in Materials and Methods. Following two weeks of puromycin selection, cells were harvested, extracted, and equal amounts of protein were subjected to western blot analysis with anti-HA (A) or anti-β-catenin antibodies (B). The asterisk in A marks a non specific anti-HA-reactive band. C2 cells were harvested at different time points following stimulation with DM and subjected to sequential immunoblot analysis with anti-MyoD and anti-myogenin antibodies (C). Note that MyoD expression is not affected in non-differentiating β-cateninY33 overexpressing cells, while myogenin expression is already suppressed (compare c to βY33 at time 0). (D) Transcriptional activity of a LEF-driven reporter in C2 cells overexpressing β-catenin. Control (c) and β-cateninY33-overexpressing C2 myoblasts (βY33) were transfected with TOPFLASH, and luciferase activity was determined. Note that luciferase activity is only slightly increased in β-catenin-overexpressing cells.

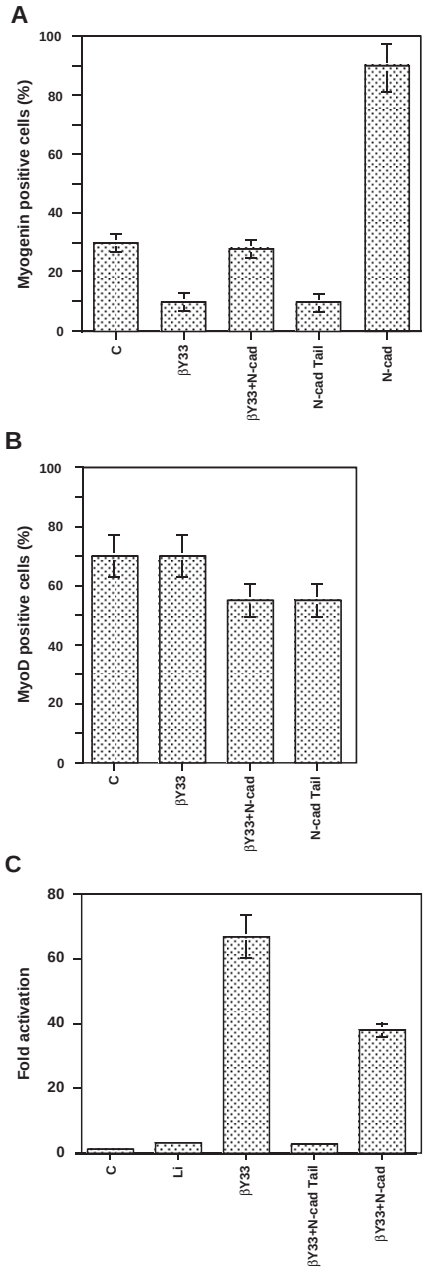

Fig. 6. Transient elevation of β-catenin levels suppresses myogenin expression. Confluent C2 cells were transiently transfected with HA-β-cateninY33 (βY33), GFP-N-cadherin tail (N-cad Tail), or full-length chicken N-cadherin (N-cad), either alone or together with HA-β-cateninY33 (βY33+N-cad). The cells were further incubated with DM for 2 days, fixed and double immunolabeled with anti-myogenin or anti-MyoD antibodies. The histograms show the difference between the fraction of myogenin-positive (A) or MyoD-positive (B) transfected cells, compared to control untransfected (c) myogenin or MyoD expressing cells, as detected microscopically. Between 100 to 150 transfected cells, out of a total of about 1000 cells (in 5-6 independent fields) were analyzed in each case. Note the decrease in the proportion of myogenin-positive cells that overexpress β-catenin or N-cadherin tail, but not in cells expressing the full length N-cadherin. (C) Transcriptional activity of LEF-driven reporter (TOPFLASH) in C2 cells treated with 30 mM LiCl (Li) or transfected with β-catenin and N-cadherin derivatives. C2 cells were co-transfected with TOPFLAH, LEF-1and βY33 either alone or together with N-cad Tail (βY33+N-cadTail) or N-cad (βY33+N-cad). Luciferase activity was assayed 48 hours after transfection in duplicates, and normalized to the control cells that were transfected with TOPFLAH and LEF-1 only (c).

myogenin level compared to controls, suggesting that an increase in adherens junction formation due to excess N-cadherin is stimulatory for myogenic progression. Based on these findings, it was concluded that the inhibitory effect of β-catenin on myogenesis can be attributed to its capacity to downregulate adherens junctions in these cells, and not to its LEF-dependent transactivation capacity. Indeed, immunofluorescent analysis confirmed that in myocytes transfected with β-cateninY33, or the N-cadherin tail, junctional localization of plakoglobin (Fig. 7, a-c and d-f) and cadherin (not shown) is severely perturbed. On the other hand, overexpression of N-cadherin either alone, or in conjunction with β-catenin, induced an increase in adherens junction formation, as shown by anti-cadherin and anti-plakoglobin, or anti-β-catenin labeling (Fig. 7, g-l).

In view of the limited capacity of N-cadherin to suppress β-catenin/LEF-1 transcriptional activity, and its ability to stimulate adherens junction formation and myogenin expression, it appears that the stimulation of myogenin expression and progression of myogenesis are triggered by adherens junction-mediated signaling rather than by suppression of β-catenin/LEF-1 driven transcription. Perturbation of adherens junctions by excess β-catenin, therefore, can inhibit the myogenic process.

DISCUSSION

In the present study we investigated the involvement of β-catenin in the regulation of skeletal myogenesis. We show here that early stages of myogenesis are accompanied by changes in the levels and subcellular distribution of β-catenin. These changes precede the expression of muscle-specific regulatory factors, such as myogenin.

The involvement of cadherin-mediated adhesion in the process of muscle differentiation is well established (Knudsen et al., 1990; Holt et al., 1994; George-Weinstein et al., 1997; Goichberg and Geiger, 1998; Linask et al., 1998; Seghatoleslami et al., 2000). In previous studies, we showed that stimulation of cadherin-mediated adhesion enhances adherens junction formation and leads to the recruitment of β-catenin to cell-cell contact sites (Goichberg and Geiger, 1998; Levenberg et al., 1998b). This process also facilitates myogenin expression and consequently the expression of muscle-specific structural proteins and the formation of fully differentiated myotubes (Goichberg and Geiger, 1998). The mechanism underlying this cadherin-mediated signaling event remained, however, unclear. In this study we wished to elucidate the specific mechanism(s) underlying these relationships.

We demonstrate here that the onset of myogenesis is accompanied by multiple changes in the levels and organization of adherens junction components. Immunofluorescent staining for β-catenin indicated that shortly after cells are moved to differentiation-inducing medium, a

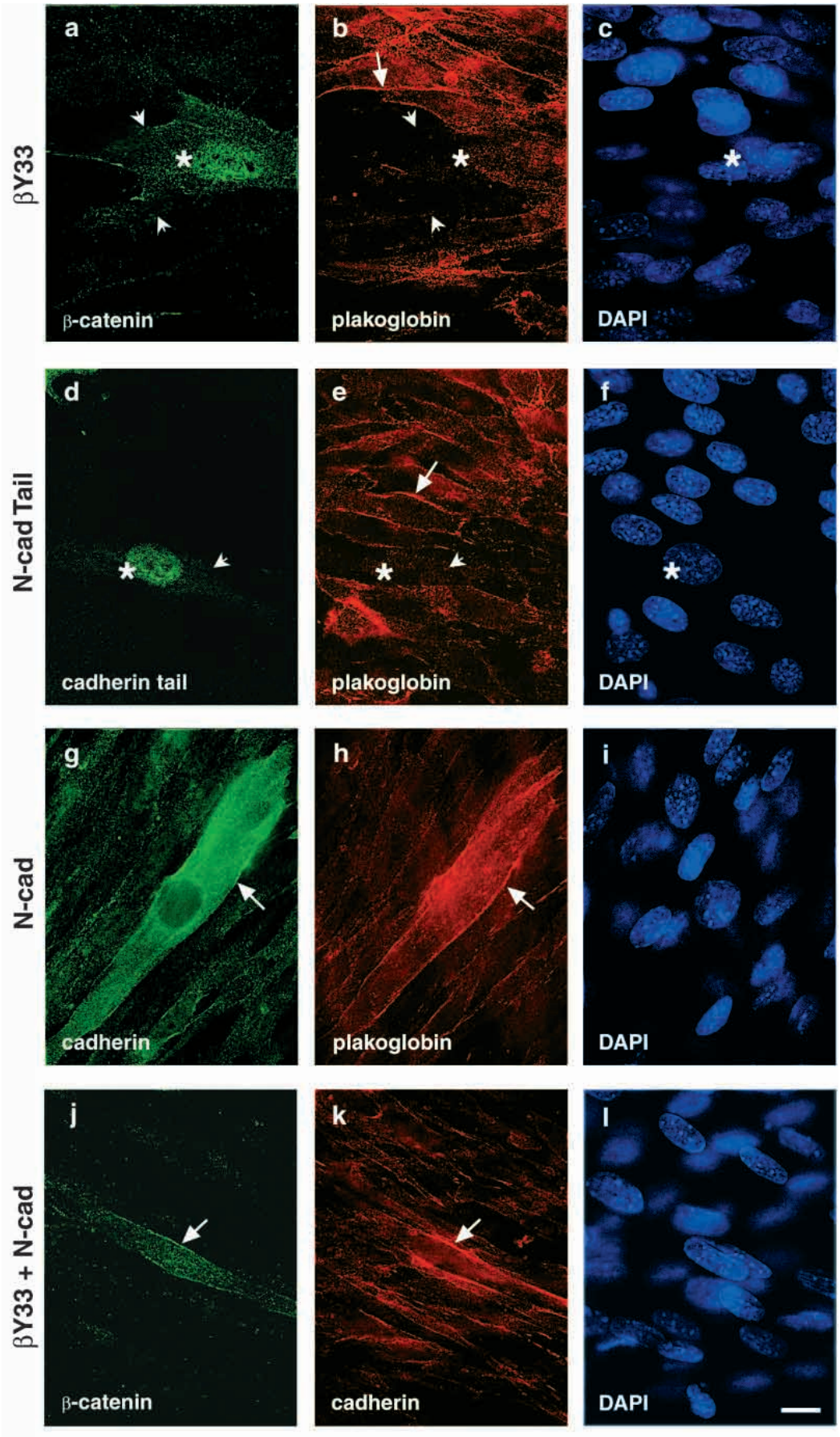

Fig. 7. The effect of β-catenin and N-cadherin derivatives overexpression on cadherin-mediated cell-cell adhesion. C2 cells were transfected as described in Fig. 6 and double immunolabeled for plakoglobin (b,e,h) or cadherin (k). Transfected cells were detected with either anti-HA antibodies, to visualize β-catenin overexpressing cells (a and j) or anti-cadherin antibodies, to visualize cadherin-overexpressing cells (g). Cells expressing the N-cadherin tail were detected by GFP fluorescence (d). Nuclei were visualized with DAPI (c,f,i,l). Digital images were acquired. Note that β-catenin- or N-cadherin tail-transfected cells did not form adherens junctions, whereas co-expression of full length N-cadherin restored junctional localization of β-catenin. The position of the transfected cell is indicated by asterisks (a-f). Long arrows point to cell-cell contacts, while the short arrows (a-b, d-e) indicate the absence of cell-cell contacts in βY33- (a,b) and N-cad Tail- (d,e) transfected cells. Bar, 10 μm.

dramatic assembly of adherens junctions occurs. This process was already evident one hour after incubation with DM and became prominent within a few hours.

β-Catenin in C2 and L8 myoblasts is mostly nuclear and associated with the Triton-soluble fraction, while the majority of this protein becomes cytoplasmic and resistant to Triton extraction at later stages of differentiation. The total amount of β-catenin also increased considerably during this process, starting at about 4 hours after induction, and reaching maximal values between 12 and 48 hours.

Which of these changes is directly involved in the induction of myogenesis? The increase in β-catenin levels, per se, is apparently not involved in myogenic induction. In fact, the artificial elevation of β-catenin effectively blocked myogenin expression. Excessive transcriptional activity driven by β-catenin and LEF-1 could be involved in this suppression of myogenesis. However, this is unlikely, as cells treated with LiCl, transfected with β-catenin, or co-transfected with β-catenin and the N-cadherin cytoplasmic tail, which blocks β-catenin's transcriptional capacity (Sadot et al., 1998, and Fig. 6C) failed to differentiate (Figs 4 and 6A).

An alternative and more likely mechanism of regulation of myogenesis by β-catenin may involve signaling from adherens junctions as a major factor in the induction of myogenin expression. This is consistent with the finding that assembly of adherens junctions was the first manifestation of myogenic induction in these cells, and was apparent within 15 minutes after stimulation with differentiation medium. Moreover, all treatments that blocked adherens junction assembly also interfered with myogenesis. The overexpression of β-catenin or the N-cadherin cytoplasmic tail had a dominant negative effect on adherens junction assembly. It should be indicated that β-catenin effectively blocked myogenin expression over a wide range of DNA concentrations (1-4 μg per 35 mm dish). On the other hand, co-expression of full length N-cadherin, together with β-catenin, effectively restored both myogenin expression and adherens junction formation. Thus, as shown in Figs 6A and 7, co-expression of functional N-cadherin reverses the inhibitory effect of high levels of β-catenin on both myogenic differentiation and adherens junction formation. Overexpression of N-cadherin alone might even accelerate the differentiation process. This is also consistent with our previous studies showing that the application of cadherin-reactive beads considerably enhances both adherens junction formation and myogenin expression (Goichberg and Geiger, 1998). Altogether, our results imply that changes in the level of β-catenin protein have a major impact on the formation of functional adherens junctions, which, in turn, is critical for myogenic differentiation. Once the balance between the amounts of β-catenin and cadherin is disturbed (as in case with β-catenin overexpression), formation of cadherin-mediated cell-cell contacts is impaired resulting in the inhibition of myogenic progression. Excess β-catenin might affect adherens junction assembly by competing for constituents of the adhesion plaque, such as α-catenin. Alternatively, it might trigger signaling cascades, of as yet unknown nature, resulting in downregulation of intercellular adhesion.

Notably, increased levels of β-catenin do not significantly affect MyoD expression and its autoregulatory activity in transfected cells, in contrast to myogenin, whose expression is dramatically reduced in such cells. Furthermore, it was previously demonstrated that calcium-dependent cell-cell interactions can stimulate myogenic differentiation without changing MyoD protein levels (Armour et al., 1999). Taken together, these data suggest that junctional integrity is essential for myogenin expression. This is further supported by the studies of Redfield et al., which demonstrated that ectopic expression of N-cadherin causes myogenic differentiation in BHK cells, while expression of a mutant N-cadherin, defective in cell-cell adhesion, fails to induce differentiation, although it is able to increase the level of β-catenin, suggesting that adherens junctions must be established in order to achieve myogenic induction (Redfield et al., 1997).

How is junctional localization of β-catenin achieved shortly after stimulation of differentiation? One possibility is that increased expression of cadherin, which accompanies early myogenic events (MacCalman et al., 1992, and Fig. 2), induces translocation of β-catenin from the cytoplasm or the nucleus to the membrane, protects it from proteasomal degradation, and induces the assembly of adherens junctions (Salomon et al., 1997; Levenberg et al., 1998a; Sadot et al., 1998; Orsulic et al., 1999). This model is, however, incompatible with the sequence of events presented here. Thus, assembly of new adherens junctions was apparent within 15 minutes to one hour, whereas a significant increase in cadherin levels was first noted only about 4 hours after the switch to differentiation medium.

Another possibility is that transmembrane signaling, triggered by the differentiation medium, induces adherens junction assembly, which, in turn, stimulates myogenin expression. Indeed, there are indications that interactions of β-catenin with various partner proteins (i.e. cadherin or α-catenin) and, consequently, its subcellular localization are affected by its phosphorylation state (Miller and Moon, 1997). In general, adherens junctions appear to be excellent candidates for such signaling processes, since they are major sites of tyrosine phosphorylation in cells (Volberg et al., 1991; Volberg et al., 1992), and they contain a large number of signaling molecules, including various kinases, their substrates and adaptor molecules (for review see Geiger et al., 1995). Our study strongly suggests that myogenesis-promoting signals, primarily those affecting myogenin expression, are generated at cadherin-mediated adhesions. The exact nature of the pathway leading to such effects and its constituents, remain, however, largely unclear. Specific signaling events in adherens junctions, which could be relevant to muscle differentiation, might include the segregation of β-catenin to newly formed adhesion sites. This could reverse a putative inhibitory activity on myogenic differentiation exerted either by direct inhibition of muscle specific genes, or via the activation of genes that suppress myogenic progression, such as cyclin D1 (Rao et al., 1994; Skapek et al., 1995; Rao and Kohtz, 1995; Skapek et al., 1996; Zhang et al., 1999). Our present findings, however, show that both the increase in β-catenin-driven transactivation (by overexpression or LiCl treatment) or its suppression (with N-cadherin tail) lead to arrest of myogenesis.

Cadherin-mediated signaling might exert its effect on myogenin by elevating the levels of the p27 cdk inhibitor, which suppresses cell cycle progression (Levenberg et al., 1999). It appears that an increase in adherens junction formation upon stimulation of differentiation in myoblasts results in the inhibition of cell growth, which, in turn, stimulates myogenic differentiation. This is consistent with

recent findings showing that cdk inhibitors such as p21 (Guo et al., 1995; Halevy et al., 1995), p18 (Phelps et al., 1998), or p27 (Zabludoff et al., 1998; Leshem et al., 2000) may have a role in the interplay between growth and differentiation of myogenic cells.

Future studies will have to address the nature of the adherens junction-mediated signals that are directly involved in junction assembly and induction of myogenin expression in the developing muscle.

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REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J*. **16**, 3797-3804.

Armour, C., Garson, K. and McBurney, M. W. (1999). Cell-cell interaction modulates myoD-induced skeletal myogenesis of pluripotent P19 cells in vitro. *Exp. Cell Res*. **251**, 79-91.

Ben-Ze'ev, A. (1997). Cytoskeletal and adhesion proteins as tumor suppressors. *Curr. Opin. Cell Biol*. **9**, 99-108.

Ben-Ze'ev, A. and Geiger, B. (1998). Differential molecular interactions of β-catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol*. **10**, 629-639.

Bullions, L. C. and Levine, A. J. (1998). The role of β-catenin in cell adhesion, signal transduction, and cancer. *Curr. Opin. Oncol*. **10**, 81-87.

Damalas, A., Ben-Ze'ev, A., Simcha, I., Shtutman, M., Leal, J. F., Zhurinsky, J., Geiger, B. and Oren, M. (1999). Excess β-catenin promotes accumulation of transcriptionally active p53. *EMBO J*. **18**, 3054-3063.

Geiger, B., Bershadsky, A. and Yehuda-Levenberg, S. (1995). Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. *Acta Anat.* **154**, 46-62.

George-Weinstein, M., Gehart, J., Blitz, J., Simak, E. and Knudsen, K. A. (1997). N-cadherin promotes the commitment and differentiation of skeletal muscle precursor cells. *Dev. Biol*. **185**, 14-24.

Goichberg and Geiger, B. (1998). Direct involvement of N-cadherin-mediated signaling in muscle differentiation. *Mol. Biol. Cell* **9**, 3119-3131.

Gumbiner, B. M. (1998). Propagation and localization of Wnt signaling. *Curr. Opin. Genet. Dev*. **8**, 430-435.

Guo, K., Wang, J., Andres, V., Smith, R. C. and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol. Cell Biol*. **15**, 3823-3829.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D. and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

He, T. C., Chan, T. A., Vogelstein, B. and Kinzler, K. W. (1999). PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* **99**, 335-345.

Holt, C. E., Lemaire, P. and Gurdon, J. B. (1994). Cadherin-mediated cell interactions are necessary for the activation of MyoD in *Xenopus* mesoderm. *Proc. Nat. Acad. Sci. USA* **91**, 10844-10848.

Hsu, S. C., Galceran, J. and Grosschedl, R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with β-catenin. *Mol. Cell Biol*. **18**, 4807-4818.

Knudsen, K. A., Myers, L. and McElwee, S. (1990). A role for the Ca^{2+}-dependent adhesion molecules, N-cadherin, in myoblast interaction during myogenesis. *Exp. Cell Res.* **188**, 175-184.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a β-catenin-Tcf complexes in $APC^{-/-}$ colon carcinoma. *Science* **275**, 1784-1787.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680-685.

Leshem, Y., Spicer, D. B., Gal-Levi, R. and Halevy, O. (2000). Hepatocyte growth factor (HGF) inhibits skeletal muscle cell differentiation: A role for the bHLH protein twist and the cdk inhibitor p27. *J. Cell Physiol*. **184**, 101-109.

Levenberg, S., Katz, B. Z., Yamada, K. M. and Geiger, B. (1998a). Long-range and selective autoregulation of cell-cell or cell-matrix adhesions by cadherin or integrin ligands. *J. Cell Sci*. **111**, 347-357.

Levenberg, S., Sadot, E., Goichberg, P. and Geiger, B. (1998b). Cadherin-mediated transmembrane interactions. *Cell Adhes. Commun*. **6**, 161-170.

Levenberg, S., Yarden, A., Kam, Z. and Geiger, B. (1999). p27 is involved in N-cadherin-mediated contact inhibition of cell growth and S-phase entry. *Oncogene* **18**, 869-876.

Linask, K. K., Ludwig, C., Han, M. D., Liu, X., Radice, G. L. and Knudsen, K. A. (1998). N-cadherin/catenin-mediated morphoregulation of somite formation. *Dev. Biol*. **202**, 85-102.

MacCalman, C., Bardeesy, N., Holland, P. C. and Blaschuk, O. W. (1992). Noncoordinated developmental regulation of N-cadherin, N-CAM, integrin, and fibronectin mRNA levels during myoblast terminal differentiation. *Dev. Dynam*. **195**, 127-132.

Mann, B., Gelos, M., Siedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Riecken, E. O., Buhr, H. J. and Hanski, C. (1999). Target genes of β-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Nat. Acad. Sci. USA* **96**, 1603-1608.

Mege, R. M., Goudou, D., Diaz, C., Nicolet, M., Garcia, L., Geraud, G. and Rieger, F. (1992). N-cadherin and N-CAM in myoblast fusion: compared localization and effect of blockage by peptides and antibodies. *J. Cell Sci*. **103**, 897-906.

Miller, J. R. and Moon, R. T. (1997). Analysis of the signaling activities of localization mutants of β-catenin during axis specification in Xenopus. *J Cell Biol*. **139**, 229-243.

Morin, P. J., Sparks, A., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 11787-11789.

Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M. and Byers, S. W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of β-catenin. *J. Biol. Chem*. **272**, 24735-24738.

Orsulic, S., Huber, O., Aberle, H., Arnold, S. and Kemler, R. (1999). E-cadherin binding prevents β-catenin nuclear localization and β-catenin/LEF-1-mediated transactivation. *J. Cell Sci*. **112**, 1237-1245.

Phelps, D. E., Hsiao, K. M., Li, Y., Hu, N., Franklin, D. S., Westphal, E., Lee, E. Y. and Xiong, Y. (1998). Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol. Cell Biol*. **18**, 2334-2343.

Porfiri, E., Rubinfeld, B., Albert, I., Hovanes, K., Waterman, M. and Polakis, P. (1997). Induction of a β-catenin-LEF-1 complex by wnt-1 and transforming mutants of β-catenin. *Oncogene* **15**, 2833-2839.

Rao, S. S., Chu, C. and Kohtz, D. S. (1994). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol. Cell Biol*. **14**, 5259-5267.

Rao, S. S. and Kohtz, D. S. (1995). Positive and negative regulation of D-type cyclin expression in skeletal myoblasts by basic fibroblast growth factor and transforming growth factor β. A role for cyclin D1 in control of myoblast differentiation. *J. Biol. Chem*. **270**, 4093-4100.

Redfield, A., Nieman, M. T. and Knudsen, K. A. (1997). Cadherins promote skeletal muscle differentiation in three-dimensional cultures. *J. Cell Biol*. **138**, 1323-1331.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. and Polakis, P. (1997). Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science* **275**, 1790-1792.

Sadot, E., Simcha, I., Shtutman, M., Ben-Ze'ev, A. and Geiger, B. (1998). Inhibition of β-catenin-mediated transactivation by cadherin derivatives. *Proc. Nat. Acad. Sci. USA* **95**, 15339-15344.

Salomon, D., Sacco, P. A., Roy, S. G., Simcha, I., Johnson, K. R., Wheelock, M. J. and Ben-Ze'ev, A. (1997). Regulation of β-catenin levels and

localization by overexpression of plakoglobin and inhibition of the ubiquitin-proteasome system. *J. Cell Biol*. **139**, 1325-1335.

Seghatoleslami, M. R., Myers, L. and Knudsen, K. A. (2000). Upregulation of myogenin by N-cadherin adhesion in three-dimensional cultures of skeletal myogenic BHK cells [In Process Citation]. *J. Cell Biochem*. **77**, 252-264.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Nat. Acad. Sci. USA* **96**, 5522-5527.

Simcha, I., Shtutman, M., Salomon, D., Zhurinsky, J., Sadot, E., Geiger, B. and Ben-Ze'ev, A. (1998). Differential nuclear translocation and transactivation potential of β-catenin and plakoglobin. *J. Cell Biol*. **141**, 1433-1448.

Skapek, S. X., Rhee, J., Spicer, D. B. and Lassar, A. B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267**, 1022-1024.

Skapek, S. X., Rhee, J., Kim, P. S., Novitch, B. G. and Lassar, A. B. (1996). Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol. Cell Biol*. **16**, 7043-7053.

Stambolic, V., Ruel, L. and Woodgett, J. R. (1996). Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells [published erratum appears in *Curr. Biol.* (1997) **7**, 196]. *Curr Biol*. **6**, 1664-1668.

Tetsu, O. and McCormick, F. (1999). β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-799.

Volberg, T., Geiger, B., Dror, R., Zick, Y. (1991). Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regul*. **2**, 105-120.

Volberg, T., Zick, Y., Dror, R., Sabanay, I., Gilon, C., Levitzki, A., Geiger, B. (1992). The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J*. **11**, 1733-42.

Willert, K. and Nusse, R. (1998). β-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev*. **8**, 95-102.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol*. **14**, 59-88.

Xu, L., Corcoran, R. B., Welsh, J. W., Pennica, D. and Levine, A. J. (2000). WISP-1 is a Wnt-1- and β-catenin-responsive oncogene. *Genes Dev*. **14**, 585-595.

Yaffe, D. and Saxel, O. (1977a). A myogenic cell line with altered serum requirements for differentiation. *Differentiation* **7**, 159-166.

Yaffe, D. and Saxel, O. (1977b). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.

Yun, K. and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Opin. Cell Biol*. **8**, 877-889.

Zabludoff, S. D., Csete, M., Wagner, R., Yu, X. and Wold, B. J. (1998). p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. *Cell Growth Differ*. **9**, 1-11.

Zhang, J. M., Wei, Q., Zhao, X. and Paterson, B. M. (1999). Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4. *EMBO J*. **18**, 926-933.