Fusion Competence of Myoblasts Rendered Genetically Null for N-Cadherin in Culture

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Abstract. Myoblast fusion is essential to muscle tissue development yet remains poorly understood. N-cadherin, like other cell surface adhesion molecules, has been implicated by others in muscle formation based on its pattern of expression and on inhibition of myoblast aggregation and fusion by antibodies or peptide mimics. Mice rendered homozygous null for N-cadherin revealed the general importance of the molecule in early development, but did not test a role in skeletal myogenesis, since the embryos died before muscle formation. To test genetically the proposed role of N-cadherin in myoblast fusion, we successfully obtained N-cadherin null primary myoblasts in culture. Fusion of myoblasts expressing or lacking N-cadherin was found to be equivalent, both in vitro by intracistronic complementation of lacZ and in vivo by injection into the muscles of adult mice. An essential role for N-cadherin in mediating the effects of basic fibroblast growth factor was also excluded. These methods for obtaining genetically homozygous null somatic cells from adult tissues should have broad applications. Here, they demonstrate clearly that the putative fusion molecule, N-cadherin, is not essential for myoblast fusion.

EVIDENCE that N-cadherin, a calcium-dependent, cell surface adhesion molecule, plays a role in myoblast fusion is drawn from its spatial and temporal pattern of expression (8, 10, 14, 19, 22) and from blocking studies either with antibodies specific to extracellular domains of N-cadherin or with peptides designed to mimic the homophilic binding site conserved among various cadherins (18, 22). A major caveat of studies using such blocking agents (18, 22, 23, 32, 40) is that the results may derive from indirect effects: nonspecific binding to other molecules or antagonistic activity such as steric hindrance that prevents physical approximation. Alternatively, such blocking agents can have agonistic or other secondary effects of ligand binding such as initiation of signal transduction (6, 24, 34). Genetic studies in which the molecule of interest is entirely eliminated have at times overcome these problems (4, 5, 35). However, mice lacking N-cadherin exhibit severe defects in neurulation, somitogenesis, and development of the myocardium, dying as 9-d embryos before muscle tissue formation (30). In some cases, such lethality has been overcome by producing chimeric mice, created by implanting embryonic stem (ES)[1] cells homozygous for a mutation of interest into wild-type blastocysts (9, 39). Tissues analyzed from a significant number of chimeric mice can be informative about the function of the mutated gene, especially if the proportion of null cells is skewed: a high proportion suggests the molecule is nonessential, whereas a low proportion suggests it is required. Unfortunately in the case of N-cadherin, selection of null ES cells necessary for the generation of chimeras has been unsuccessful to date. Thus, a genetic analysis of N-cadherin function in skeletal myogenesis has not previously been possible.

To overcome these problems and test genetically the role of N-cadherin in skeletal muscle fusion in vitro and in vivo, we undertook the novel approach of selecting myoblasts that were genetically null for N-cadherin. In this report, we show that myoblasts lacking or expressing N-cadherin fuse equivalently both in culture and in the muscles of adult mice, thus ruling out an essential role for N-cadherin in the process of myoblast fusion.

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1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; ES, embryonic stem.

Materials and Methods

G418 Selection of Homozygous Primary Myoblasts Lacking N-Cadherin

A heterozygous N-cadherin–deficient male mouse was created by homologous recombination (30) and was mated to a wild-type C57 BL/6 female. Primary myoblasts were isolated and purified, as described (31) from a litter of eight 1-wk-old pups. Myoblasts were maintained in growth medium (GM) as in (31) except that 40% Ham's F10 and 40% DME were used and G418 (0.2 mg/ml) was included in the medium until myoblasts had grown to $\sim 2 \times 10^7$ cells. At this point they were plated at a subconfluent density of $\sim 5 \times 10^5$ cells/150-mm culture dish and subjected to stringent selection in G418 (5 mg/ml) for 3 wk in GM buffered to pH 7.2 with 10 mM Hepes. Of 96 clones isolated and transferred to a microwell plate, 6 grew significantly after 3 wk of further selection. Genomic DNA was isolated from these clones, ethanol precipitated, digested with EcoRV, and screened for wild-type or disrupted N-cadherin alleles by Southern hybridization as described (30). Null clones were further subcloned to assure homozygosity. For Western blot analysis, myoblasts were maintained in GM or grown for 4 d in differentiation medium (DM) as in (31), except that 5% horse serum was used. Cells were lysed and 20 μg protein from myoblasts or myotubes of each clone was electrophoresed and transferred to PVDF membrane (Millipore Corp., Bedford, MA). Blots containing replicate samples were probed with (*a*) MNCD-2, a rat monoclonal antibody to a fusion protein containing amino acids 308–597 specific to the extracellular domain of mouse N-cadherin (20); (*b*) with mouse monoclonal antibody (C1821; Sigma Chemical Co., St. Louis, MO) to a synthetic peptide corresponding to the COOH-terminal 24 amino acids of chicken N-cadherin; or (*c*) with anti-pan cadherin, a rabbit polyclonal antibody made to a GST fusion to the entire COOH-terminal domain of E-cadherin, a domain that is highly conserved among most members of the cadherin family of molecules including M-, R-, and N-cadherins (generous gift of W.J. Nelson, Stanford University, Stanford, CA). Antibody binding was detected with HRP-conjugated sheep anti–rat IgG (Sigma Chemical Co.), sheep anti–mouse IgG, or donkey anti–rabbit IgG and enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL).

Chemiluminescent Assay

Separate populations of N-cadherin–lacking (−/−) or N-cadherin–expressing (+/−) myoblasts were transduced with retroviral constructs encoding nonfunctional complementing mutant peptides (Δω and Δμ) of the β-galactosidase enzyme as described (25). Cells expressing one peptide were mixed at equal densities with cells of the same type expressing the complementing peptide, allowed to attach overnight in 96-well microplates, after which GM was changed to DM with or without recombinant human basic fibroblast growth factor (bFGF; Promega, Madison, WI) and replaced daily. At the indicated time points, cells were lysed, processed as described (25) for chemiluminescent assay using the Galacton-Plus chemiluminescent detection kit (Tropix, Bedford, MA), and enzyme activity was measured with a luminometer (MicroBeta 1450; Wallac Inc., Gaithersburg, MD). Data are expressed as the means of eight replicate wells ±SD. In some experiments, heterozygous clones were compared to homozygous null ones since they most resembled null clones in strain background and passage number, factors known to affect myoblast fusion.

Fusion Index

Cells assayed for fusion index were treated identically to those in chemiluminescent assays except that they were fixed, stained with methylene blue, and random fields photographed. Each value derived from four independent fields scored at 250×. Standard error of the proportion was computed as $\sqrt{\hat{p}(1-\hat{p})/n}$, where $\hat{p}$ is the estimated proportion of the whole population of nuclei found in myotubes at a given time point.

Tricolor Fluorescent Histochemistry

Populations of myoblasts were labeled with complementing segments of the *lacZ* gene as described (25), mixed together in equal portions, and plated on sterile collagen-coated glass coverslips. Fusing cells were fixed for 4 min in 4% paraformaldehyde and then blocked in PBS with 10% horse serum. β-galactosidase activity was detected with 25 μg/ml Fast red violet-LB salt (Sigma Chemical Co.) plus 100 μg/ml 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Fluka Chemika-Bio Chemika, Buchs, Switzerland) in PBS after labeling nuclei with DAPI (Sigma Chemical Co.) and myotube membranes with rat anti-NCAM antibody (Chemicon International, Inc., Temecula, CA; 10, 19, 22), biotinylated goat anti–rat secondary antibody (Vector Laboratories, Burlingame, CA), and Cy5-labeled streptavidin (Amersham Corp.). Triple-labeled deconvolved images were collected using a deconvolution microscope (Delta Vision; Applied Precision Inc., Mercer Island, WA).

Transplantation of β-Galactosidase–Labeled Cells into Muscles of Nude Mice

Primary myoblasts were transduced with a retroviral construct, MFG KB5 containing the full length *lacZ* gene (25), exposed to the substrate fluorescein di-β-D-galactopyranoside (Molecular Probes, Eugene, OR) and cells expressing β-galactosidase isolated with the fluorescence-activated cell sorter as described (28). Injections of β-galactosidase–positive cells into nude mice (two injections/leg; 5×10^5 cells/injection) were as described (31). After 2 or 12 d, mice were killed and muscle dissected, cryofrozen, sectioned, stained as described (31), and photographed using differential interference optics.

Results

G418 Selection of Homozygous Primary Myoblasts Lacking N-Cadherin

A phenotypically normal adult mouse heterozygous for a targeted disruption of the N-cadherin gene (30) was mated with a wild-type mouse. Primary myoblasts from a mixed litter of heterozygous N-cadherin–deficient and wild-type pups were pooled, enriched to eliminate nonmyogenic cell types, and grown to high numbers according to previously published methods (31). The cells were then exposed to low G418 selection media, a treatment that yielded a relatively pure population of heterozygous cells as indicated by a shift in the ratio of wild-type to mutant alleles from ~2:1 to 1:1 by Southern analysis (Fig. 1 *A*). To select for homozygous mutant myoblasts, this heterozygous myoblast population was then subjected to more stringent selection in high G418. Sparse plating of cells was critical in this procedure as G418 is only selective against actively growing cells. A dose-response curve revealed a requirement for a significantly higher concentration of G418 to kill most unwanted heterozygous myoblasts than that previously reported for ES cells (26), yet the efficiency of recovery of clones was in the same range. Most clones grew very slowly in this concentration of G418. Of the six that formed large-size colonies and were assayed for conversion to homozygosity, two (H7 and G12) were found to harbor only mutant N-cadherin alleles by Southern analysis (Fig. 1 *B*). Probing of Western immunoblots with either a monoclonal antibody that specifically recognizes the extracellular domain of N-cadherin or with a monoclonal antibody to the COOH-terminal 24 amino acids of N-cadherin demonstrated detectable protein of the proper molecular weight in wild-type cultures but not in null clones. By contrast, a Western blot of these same samples probed with a polyclonal antibody to the full COOH-terminal domain that is highly conserved among most members of the cadherin family including M- and R-cadherin revealed other cadherin protein(s) on N-cadherin null myoblasts and myotubes (Fig. 1 *C*). Although cells of the H7 null clone exhibited somewhat rounded morphology and slow growth, cells of one heterozygous clone did as well. Thus, these characteristics can be attributed to clonal vari-

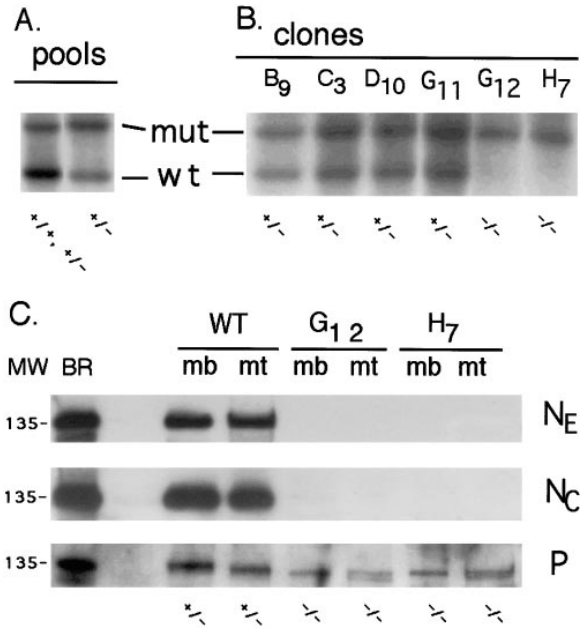

Figure 1. G418 selection of homozygous primary myoblasts lacking N-cadherin. (*A*) Southern blot analysis of a population of primary myoblasts isolated and purified from leg muscles of progeny of a wild-type X heterozygous cross reveals a mixture of homozygous wild-type and heterozygous cells (lane *1*: +/+, +/−) that were rendered purely heterozygous (lane *2*: +/−) after growth in low-dose G418. (*B*) Southern blot analysis of six clones of primary myoblasts (B9, C10, D3, G11, G12, H7) after selection in high-dose G418 demonstrates that clones G12 and H7 myoblasts have been rendered genetically homozygous null for N-cadherin. (*C*) Western blot analysis of wild-type, G12, and H7 cells with antibodies that specifically recognize the extracellular domain (N_E) or that recognize the COOH-terminal 24 amino acid sequence (N_C) of N-cadherin confirms that the genetically null G12 and H7 clones lack N-cadherin protein both as undifferentiated myoblasts (*mb*) and as syncytial myotubes (*mt*). Probing of same samples with a polyclonal antibody made to the entire highly conserved COOH-terminal domain common to many cadherins (*P*) shows that N-cadherin null clones clearly express some other member of the cadherin family of cell adhesion proteins. Lysed mouse brain tissue (*BR*) served as control for the 135-kD N-cadherin protein.

ation rather than lack of N-cadherin surface protein. By microscopic analysis, all heterozygous and homozygous null clones fused extensively.

Quantitation of Fusion Potential of N-Cadherin–Expressing or Null Myoblasts In Vitro

We assessed the fusion potential of N-cadherin–expressing and -nonexpressing myoblasts in tissue culture. Fusion was measured using chemiluminescence by adapting a recently developed method based on intracistronic complementation of the *lacZ* gene in mammalian cells (25). Briefly, as applied here, the assay involved infecting replicate primary muscle cell cultures with retroviruses that encoded one of two nonfunctional mutant β-galactosidase peptides. Functional enzyme was only produced upon myoblast fusion, at which point the two peptides were able to come in contact, assemble, and complement one another. Shown for the first time (Fig. 2 *A*) is evidence that this quantitative biochemical assay yields results in good agreement with those obtained by the slower, more labor-intensive microscopic scoring of fusion index. In addition, the activity of complemented β-galactosidase was assayed in situ with a novel fluorescent substrate, and when visualized together with other fluorescent markers of nuclei and myotube membranes, clearly demonstrated fusion at the single cell level (Fig. 2 *B*). Conclusive evidence for differentiation was provided by the observation that the N-cadherin null myofibers actively contracted in culture. Thus, data from both biochemical and microscopic assays of fusion were in good agreement and showed that the kinetics and extent of fusion were similar for myoblasts expressing or totally lacking N-cadherin.

Fusion Potential of N-Cadherin–Expressing and Null Myoblasts In Vivo

To determine whether N-cadherin is essential for fusion to occur in vivo, we injected mutant myoblasts into the muscles of adult mice and monitored their fusion with preexisting host muscle fibers (29, 31). These experiments were of particular importance, since the fusion behavior of myoblasts in vitro can differ markedly from that in vivo; see for example, studies of myogenin null myoblasts (13, 27). Myoblasts were marked by infection with a replication-defective retrovirus encoding the full-length β-galactosidase enzyme and injected into the tibialis anterior muscles of nude mice (31). Fusion of homozygous null (−/−), heterozygous (+/−), and wild-type (+/+) myoblasts with muscle fibers was indistinguishable when visualized histologically. In all cases, irrespective of N-cadherin expression, 2 d after injection unfused myoblasts were found in clusters (Fig. 3, *top left*), whereas 12 d after injection, myoblasts were localized in fields of hundreds of large diameter β-galactosidase–containing fibers (Fig. 3, remaining images). These results demonstrate that N-cadherin is not required for myoblasts to gain access to host fibers and fuse into pre-existing syncytia of adult muscles.

Effect of Basic Fibroblast Growth Factor on Fusion of N-Cadherin Null Myoblasts

Several cell adhesion molecules, including N-cadherin, share a homologous domain with the FGF receptor (38), and cell adhesion molecules generally have been implicated in signal transduction pathways, some of which involve myogenic migration and differentiation (1, 3, 11, 12, 15, 21). Indeed, N-cadherin has been shown to activate a second messenger pathway involving a tyrosine kinase that is similar, if not identical, to that activated by bFGF in neurons (37). To test genetically whether N-cadherin is involved in mediating effects of bFGF on myoblast differentiation we employed the rapid biochemical assay of fusion described above based on β-galactosidase complementation. The dose-response curve exhibited by N-cadherin null and N-cadherin–expressing myoblasts was similar and led to half maximal inhibition of fusion at 0.45 ng/ml bFGF in good agreement with previous microscopic assays of wild-type primary myoblasts (31) (Fig. 4). These results demonstrate that N-cadherin expression in vitro is not essential for transduction of the bFGF signal to myoblasts.

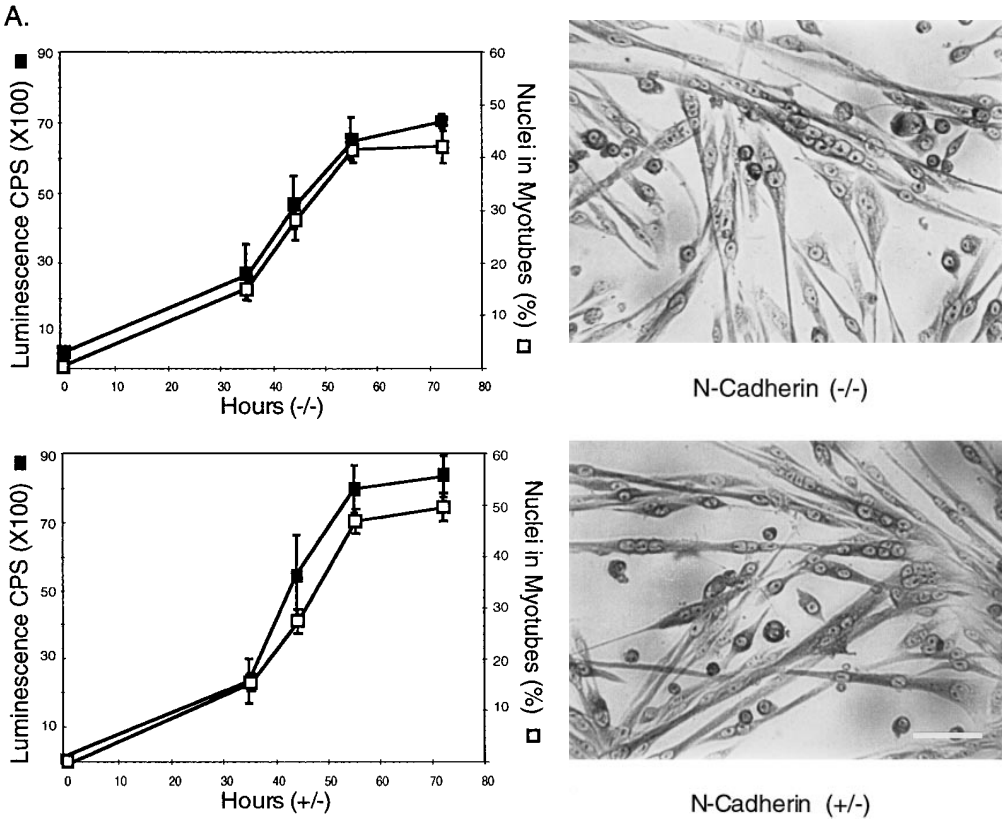

Figure 2. Similar kinetics and extent of fusion of primary myoblasts expressing or lacking N-cadherin. (*A*; *graphs*) A biochemical chemiluminescence assay of fusion gave similar results to the fusion index, or percentage of total nuclei in myotubes. (A; *greyscale images*) Micrographs of fusing N-cadherin +/− or −/− myoblasts after 55 h in DM. (*B*) Activity of functional complemented β-galactosidase in fused cells detected at the single cell level by fluor-X-gal (*green*) together with nuclei (*blue*) and myotube membrane marker (*red*). Bars: (*A*) 50 μm; (*B*) 10 μm.

Discussion

The ability to produce homozygous null somatic cells in culture allows a genetic analysis of the function of molecules in differentiated cells in a manner that is not always possible in whole animals. This is particularly clear in cases in which the null mutation of interest results in embryonic lethality before the development of the tissue of interest, as in the case of N-cadherin. Viability as a cell monolayer in vitro presumably does not impose the same stringent selective pressures as are required for viability and development of an intact animal, thus functional compensation by other molecules may occur less frequently. Therefore, application of a genetic approach to cultured somatic cells should prove useful in some cases in which no phenotype is observed in the mouse. In either case, genotype conversion in vitro could provide an indication of the function of particular molecules that may be missed by targeted gene inactivation in the whole animal.

The approach for obtaining homozygous null somatic cells described in this report is a modification of that previously reported for ES cells (26). The precise molecular mechanism by which most heterozygous ES cells or the N-cadherin heterozygous myoblasts described here are rendered null, remains unknown. However, since no phenotype was apparent in N-cadherin null myoblast clones, major disruptions such as chromosomal loss or duplication seem less likely than homologous recombination at mitosis

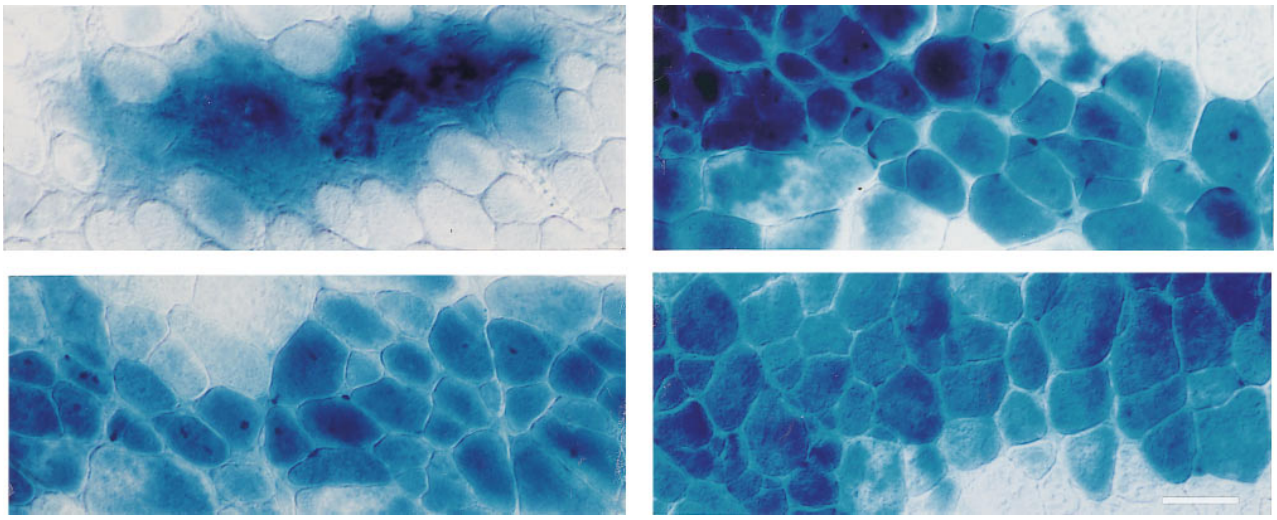

Figure 3. Fusion of N-cadherin homozygous null, heterozygous, and wild-type myoblasts with multinucleate myofibers in vivo. N-cadherin −/−, +/−, and +/+ primary myoblasts were each transduced with retroviral constructs containing the full-length *lacZ* gene and then injected into the tibialis anterior muscles of nude mice. At 2 d, myoblasts were present in unfused clusters (shown here for +/+, *upper left*). By 12 d after injection for all phenotypes (+/+, *top right*, +/−, *bottom left*, and −/−, *bottom right*) *lacZ* expression was also similar but appeared in fields of hundreds of large-diameter muscle fibers indicative of fusion with pre-existing host fibers. Bar, 60 μm.

leading to increased expression of the neo gene used for selection.

The process by which muscle cells interact and fuse to produce syncytia has been studied for decades and yet remains largely unknown. Myoblasts of different species recognize one another, adhere and fuse to form heterokaryon myotubes, yet very rarely spontaneously fuse with cells from other tissues (2). A number of molecules have been implicated in the adhesion and fusion of myoblasts, including N-, M-, and R-cadherin, neural cell adhesion molecule (NCAM), vascular cell adhesion molecule (VCAM-1), meltrin, and various integrins (7, 18, 22, 23, 32, 38, 40). In some cases, these molecules were thought to be involved due to increased fusion observed upon over-expression (7, 40). In other cases, both inhibition of aggregation and fusion were documented in response to antibodies or peptide mimics (17, 18, 22, 23, 32, 40). The generation of transgenic animals with targeted deletions in genes for a couple of these molecules, N-cadherin and α-4 integrin (30, 41), recently allowed homozygous null myoblasts to be isolated from mouse tissues and selected after growth in G418 in tissue culture. Using a genetic approach, α-4 integrin, like N-cadherin shown here, has proven not to be essential to myogenesis. These findings raise questions regarding the results of previous studies that employed over-expression or blocking agents such as antibodies or peptides to prove that a molecule has a role in the fusion of myoblasts. Blocking agents may have indirect effects such as nonspecific binding, steric hindrance, and initiation of signal transduction (6, 24, 34). A genetic approach provides the most stringent test of the function of a molecule. If a gene is deleted and a process continues, the specific molecule encoded by that gene is clearly not required, as is the case for N-cadherin in myoblast fusion.

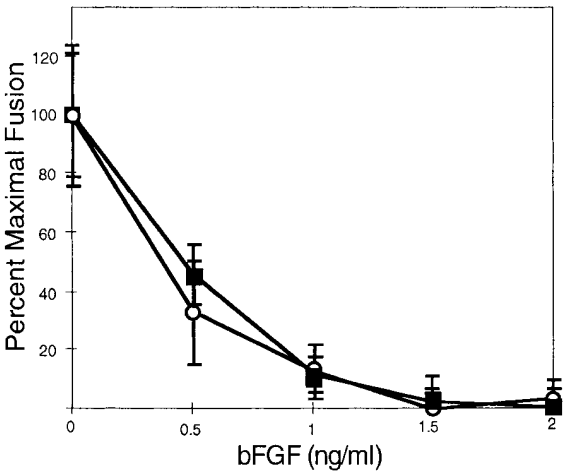

Figure 4. bFGF inhibition of fusion of N-cadherin–lacking and -expressing myoblasts. β-Galactosidase activity in 50-h fusing cocultures of homozygous N-cadherin null (○) and wild-type (■) myoblasts treated with different concentrations of bFGF in DM was measured by chemiluminescent assay. Standard deviations were calculated on data expressed as percent maximal fusion, normalized by the equation $(Y\text{-}Y_o)/Y_{max}\text{-}Y_o$, where Y is individual measure of luminescent counts per second (LCPS) for a given well, Y_o is mean LCPS value for that point, and Y_{max} is mean LCPS value at point of maximum fusion where FGF = 0 ng/ml.

However, genetic studies that lead to the elimination of a molecule such as N-cadherin do not exclude the possibility that other molecules may function in place of the targeted one (16). It has long been known that cadherins, by virtue of their homophilic binding, play a role in aggregation of like cell types (11, 35). There is also suggestive evidence that a cadherin may be important in signaling myogenic differentiation and that one cadherin may replace another in this role (15). Indeed, muscle has been reported to express several other cadherins, including M- and R-cadherins, that have been suggested to play roles in myogenesis (21). We have shown in this report that cadherins other

than N-cadherin are present on N-cadherin null myoblasts. These cadherins or other adhesion molecules could assume its role. Thus, the present study does not rule out the possibility that N-cadherin participates in aggregation, differentiation, or fusion. However, its role in these processes, if it has one, is definitely not essential.

In conclusion, targeted gene inactivation now allows a rigorous genetic analysis of the function of specific molecules, not only in developing mice, but also in the cultured somatic cells derived from their adult tissues. Thus, the approach described here for studying the role of N-cadherin in muscle cell fusion has potential for determining the importance of diverse molecules to the process of myogenesis and could well be extended to a range of other somatic cell types.

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