

Hepatocyte Growth Factor (HGF) Inhibits Skeletal Muscle Cell Differentiation: A Role for the bHLH Protein Twist and the cdk Inhibitor p27

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Hepatocyte growth factor (HGF) plays a crucial role in regulating the differentiation of both fetal and adult skeletal myoblasts. This study aimed at defining the intracellular factors that mediate the effect of HGF on adult myoblast differentiation. HGF increased Twist expression while decreasing p27^{Kip1} protein levels and not affecting the induction of p21^{Cip1/Waf1} in satellite cells. Like HGF, overexpression of Twist did not affect p21 expression while inhibiting muscle-specific proteins. Both ectopic Twist-antisense (Twist-AS) and p27 partially rescued the effects of HGF on bromodeoxyuridine (BrdU) incorporation and myosin heavy chain (MHC) expression in muscle satellite cells; the two plasmids together effected full rescue, suggesting that HGF independently regulates these two factors to mediate its effects. Ectopic p27 promoted differentiation in the presence of HGF by blocking the induction of Twist. Using Twist-AS to lower Twist levels restored the HGF-dependent reduction of p27 and MHC. In the presence of ectopic HGF, satellite cells formed thin mononuclear myotubes. Neither ectopic p27, Twist-AS, or their combination reversed this change in cell morphology, suggesting that HGF acts through additional mediators to inhibit downstream events during myogenesis. Taken together, the results suggest that the effects of HGF on muscle cell proliferation and differentiation are mediated through changes in the expression levels of the myogenic-inhibitory basic helix-loop-helix (bHLH) protein Twist and the cell-cycle inhibitor p27. *J. Cell. Physiol.* 184: 101–109, 2000. © 2000 Wiley-Liss, Inc.

The multiplication of myogenic cells plays a crucial role during growth and injury repair in adult skeletal muscle, and is solely dependent on a unique group of cells, the satellite cells (also called adult myoblasts). These small mononucleate cells lie mostly in a quiescent state under the basal lamina of the myofiber and are uniformly distributed throughout the length of the muscle (Campion, 1984). During muscle growth or upon injury, satellite cells become mitotically active, displaying programmed proliferation and differentiation followed by the expression of muscle-specific proteins (Bischoff, 1994). Many different growth factors have been shown to affect satellite cell proliferation and differentiation in culture; however, the best candidate for this role in vivo is hepatocyte growth factor (HGF), also known as scatter factor.

HGF is a multifunctional cytokine that has pleiotropic effects on several different cells and tissues. It serves as a mitogen and motility factor and plays an active role in liver, kidney, and lung regeneration (Stocker et al., 1987; reviewed in Michalopoulos and

DeFrances, 1997; Birchmeier and Gherardi, 1998). In addition, HGF plays a pivotal role in the development, growth, and regeneration of skeletal muscle. HGF and its receptor, c-Met, have been shown to be expressed in developing limb buds (Sonnenberg et al., 1993; Myokai et al., 1995; Thery et al., 1995); in *c-met*-null mutant mouse embryos, the limb bud is not occupied by myogenic precursor cells, thus skeletal muscle is not formed (Bladt et al., 1995). In addition, reducing the ability of c-Met to transduce its signal in vivo caused a striking reduction in limb muscles coupled with a gen-

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eralized deficit of secondary fibers (Maina et al., 1996). In adults, HGF protein is present in mature muscle fibers (Tatsumi et al., 1998) and its expression dramatically increases during regeneration following ischemic injury (Jennische et al., 1993). HGF has the unique property of being able to activate quiescent satellite cells (Allen et al., 1995; Gal-Levi et al., 1998) and has been shown to be the activating factor in extracts of crushed muscle for these cells (Tatsumi et al., 1998). Once activated, satellite cells are kept proliferating and are inhibited from differentiating by HGF both in vitro (Anastasi et al., 1997; Gal-Levi et al., 1998) and in vivo (Miller et al., 2000).

As in other differentiation programs, skeletal muscle differentiation is a multi-step process involving the coordinate regulation of changes in gene expression and an exit from the cell cycle. Expression of early genes, such as the tumor-suppressor gene p53 and the cyclin-dependent kinase (cdk) inhibitor p21, occurs just prior to the G₀ arrest, while later genes, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK), are only expressed following terminal growth arrest (Rao et al., 1994; Halevy et al., 1995; Andrés and Walsh, 1996). There are two families of cdk inhibitors: the Cip/Kip family (p21^{Cip1/Waf1}, p27^{Kip1}, and p57^{Kip2}) and the Ink4 family (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}). The regulation of cdk activity during the G₁ phase of the cell cycle functions to coordinate cell-cycle withdrawal with muscle differentiation. Overexpression of cyclin D1 in myoblasts inhibits their differentiation (Rao et al., 1994; Skapek et al., 1995, 1996), while expression of the cdk inhibitors p16, p21, and p27 promotes differentiation (Skapek et al., 1995; Zabludoff et al., 1998). Furthermore, during myogenesis, p21, p27, p57, and p18 are all upregulated coincident with and ensuring terminal growth arrest (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995; Franklin and Xiong, 1996; Guo and Walsh, 1997; Zhang et al., 1997; Zabludoff et al., 1998).

The MyoD family of basic helix-loop-helix (bHLH) transcription factors positively regulates myogenesis and acts in collaboration with MEF2 transcription factors to induce muscle-specific gene expression. The activity of these transcription factors is negatively regulated by cdk activity as well as by other HLH and non-HLH-containing proteins (reviewed in Lassar et al., 1994). One such inhibitor is the bHLH transcription factor Twist. During embryonic development, Twist is broadly expressed in the mesoderm, including muscle precursor cells, and its expression decreases as the cells differentiate (Hopwood et al., 1989; Wolf et al., 1991; Fuchtbauer, 1995; Stoetzel et al., 1995; Gitelman, 1997). Constitutive expression of Twist in cultured myoblasts inhibits differentiation (Hebrok and Fuchtbauer, 1994) by inhibiting the activities of both the MyoD and MEF2 transcription factors (Spicer et al., 1996; Hamamori et al., 1997; Hebrok et al., 1997).

Previously, we and others demonstrated that HGF is mitogenic for muscle satellite cells (Allen et al., 1995; Anastasi et al., 1997; Gal-Levi et al., 1998). Moreover, we showed that in primary chicken satellite cells, addition of recombinant human HGF or transfection of chicken HGF inhibits their differentiation (Gal-Levi et al., 1998). The purpose of this study was to evaluate the intracellular factors mediating the inhibition of skele-

tal muscle cell differentiation by HGF. The results show that Twist and p27 are regulated by HGF and suggest that both factors are required to mediate the effect of HGF on cell proliferation and differentiation.

MATERIALS AND METHODS

Cell cultures and transient transfections

Chicken skeletal muscle satellite cells were cultured from the pectoral muscle of 4- to 5-day-old chicks as described by Halevy and Lerman (1993). Cells were plated at 5×10^4 cells/cm² in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 10% (v/v) horse serum (HS), and grown for two days. On the third day, the medium was changed to one containing only DMEM, and the cells were transiently transfected with the specified plasmids (see below) using lipofectamine (GibcoBRL, Grand Island, NY) as described previously (Gal-Levi et al., 1998). Plasmids were mixed in DMEM with 10 μ l lipofectamine and added to the cells for 2 h, after which the medium was replaced with one containing 10% FCS. Cells were then grown for an additional 36 to 48 h. Transfection efficiency was approximately 40% as analyzed by cell transfection for β -galactosidase (β -gal)-encoding plasmid and in-situ staining for β -gal activity.

C2 myogenic cell-line derived from mouse satellite cells (Yaffe and Saxel, 1977), and C2-Twist, C2 cells stably expressing mouse Twist (Spicer et al., 1996), were grown in 20% (v/v) FCS-containing medium. Myogenic differentiation was induced by incubating the cells for two to three days in medium containing 2% (v/v) HS and 10 μ g/ml insulin.

Reporter assays

Cells were harvested for chloramphenicol acetyl transferase (CAT) assays as described previously (Gal-Levi et al., 1998). Transfection efficiency was normalized by correcting CAT activity for the levels of constitutively expressed β -gal plasmid (Maniatis, 1989). Luciferase assays were performed as suggested by the manufacturer (Promega, Madison, WI). Each transfection treatment was replicated three times in triplicates.

Plasmids

The pCSA-cHGF construct is described in Gal-Levi et al. (1998). MEF2x4CAT is a CAT reporter gene driven by four reiterated MEF2 sites driving the minimal *Xenopus* MyoD1 promoter (Wong et al., 1994). Full-length chicken Twist cDNA (accession number AF093816), p27 (Polyak et al., 1994), and p21 (Harper et al., 1993) were subcloned in both orientations into pCSA containing the cytomegalovirus (CMV) promoter (Skapek et al., 1995).

Western blot analysis

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40 with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin and pepstatin (10 μ g/ml each), aprotinin (20 μ g/ml), and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄). Cell extracts were sonicated using an ultrasonic cell disrupter (Microson, Farmingdale, NY), clarified by centrifugation, and frozen at -80°C . Cells

were normalized to protein content and proteins were separated by SDS-PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). After blocking with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% (v/v) Tween 20) containing 10% skim milk, membranes were incubated for 2 h at room temperature with the appropriate antibodies. Membranes were then washed with TBS-T and incubated for 1 h with horseradish peroxidase sheep anti-mouse IgG (Amersham, Bucks, UK). Proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). The following antibodies were used: polyclonal anti-p21 (1:1,000; PharMingen, San Diego, CA), monoclonal anti-p27 (1:3,000; Transduction Laboratories, Lexington, KY), a monoclonal antibody against sarcomeric myosin (1:10; MF-20; Developmental Studies Hybridoma Bank, University of Iowa; contributed by Dr. D. Fischman), and polyclonal antibodies against MEF2 and Twist (1:250; Santa Cruz Biotechnology, CA). It should be noted that Santa Cruz MEF2C rabbit antibodies were generated against human MEF2A but cross-react with both human and mouse MEF2A, MEF2C, and MEF2D proteins (data not shown).

Immunofluorescence

Cells were fixed in 2% (v/v) paraformaldehyde and immunostained as described in Halevy et al. (1995). Cells were stained for MHC using the monoclonal antibody MF-20 as a primary antibody at a dilution of 1:5, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Jackson, West Grove, PA) as the secondary antibody at a 1:250 dilution. For BrdU staining, additional fixation in 2% paraformaldehyde and DNA denaturation were performed, followed by incubation with a mouse anti-BrdU antibody diluted 1:2,000 (G3G4; Developmental Studies Hybridoma Bank, University of Iowa, contributed by Dr. D. Kaufman), then incubated with biotinylated anti-mouse IgG and Texas Red avidin D (both diluted 1:250; Jackson). Nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI) in PBS.

RNA preparation and RT-PCR

Total RNA was isolated and RT-PCR was performed as described by Münsterberg and Lassar (1995). In brief, total RNA was subjected to DNase treatment (RNase-free DNase, Boehringer Mannheim, Mannheim, Germany) for 10 min at 37°C. After incubation, samples were phenol/chloroform-extracted and ethanol-precipitated at -80°C. The RNA pellet was reverse-transcribed for 1 h at 42°C in a cocktail containing transcription buffer (GibcoBRL), 0.5 mM of each dNTP, 200 ng of random hexamer primer, 3.3 mM DTT, and 200 U of SuperScript reverse transcriptase (GibcoBRL). A 1- μ l aliquot of cDNA was used as a template for PCR amplification. After an initial denaturation step at 93°C for 3 min, the reactions were cycled at 93°C (30 s), 60°C (30 s), and 72°C (1 min) in an MJ Research thermocycler (Watertown, MA). The reaction products for MHC and Twist were amplified for 30 cycles while the product for GAPDH was amplified for 23 cycles. A 5- μ l aliquot of each PCR reaction was analyzed on a 1.5% agarose gel, followed by ethidium bromide staining. Bands were visualized by video cam-

era (Dinco-Ranium, Jerusalem, Israel). The following primers were used: MHC 5'-GATCCAGCTGAGCC-ATGCCA-3', 5'-GCTTCTGCTCAGCATCAACC-3' and Twist 5'-GCAGAGCGACGAGCTGGACTC-3', 5'-TGT-TATCTAGGGCTCTTGCCGG-3', GAPDH 5'-AGTCA-TCCCTGAGCTGAATG-3', 5'-AGGATCAAGTCCACA-ACACG-3'. The expected product sizes were MHC, 616 nt; Twist, 150 nt; GAPDH, 330 nt.

Thymidine incorporation

DNA synthesis was assessed by [³H]thymidine incorporation as described previously (Halevy and Lerman, 1993). Cells were transfected in 12-well plates, and at 40 h post-transfection, [³H]thymidine (New England Nuclear, Boston, MA) was added (6 μ Ci/well) for an additional 4 h of incubation. The cells were then detached with 0.25% (w/v) trypsin-EDTA and precipitated with 10% (w/v) trichloroacetic acid. Radioactivity in the dissolved precipitates was counted in Ultima Gold scintillation fluid (Packard, Downers Grove, IL) using a Tri-Carb 1600CA scintillation counter (Packard).

RESULTS

HGF differentially affects myogenic differentiation markers

As a first step to dissect the mechanism by which HGF inhibits differentiation, we analyzed the expression of different markers of myogenic differentiation in control primary satellite cells vs. ones that had been transfected with an expression construct for the chicken HGF gene. A transfection efficiency of 30 to 40% was achieved in all transfections. Transfection of satellite cells with HGF had very little effect on MEF2 protein levels (Fig. 1A). However, when HGF was transfected together with an MEF2 reporter construct, HGF reduced activation of the MEF2 promoter to approximately 45% of controls (Fig. 1B), in agreement with the inhibition seen in MHC expression (Fig. 1C) and the transactivation of MCK promoter (Gal-Levi et al., 1998). HGF differentially affected the expression levels of cdk inhibitors which are upregulated during muscle cell differentiation; the protein levels of p21 were unaffected by ectopic HGF (Fig. 1C). In contrast, HGF consistently caused a 50 to 60% decrease in p27 protein expression.

The disparity in HGF's effect on p27 vs. p21 was further analyzed in a proliferation assay using labeled thymidine as a marker for S-phase entry. Satellite cells were transfected with HGF alone or together with expression vehicles for p21 or p27. Forty hours post-transfection, [³H]thymidine was added to the culture for an additional 4 h before harvest. As shown in Figure 1D, ectopic HGF increased thymidine incorporation levels in satellite cells approximately 2.4-fold relative to control cells transfected with empty vector (Fig. 1D, lanes 1 and 2). Ectopic p27 substantially inhibited the HGF-induced increase in thymidine incorporation, bringing the level to 1.5-fold that of control cells (Fig. 1D, lane 3). In contrast, ectopic p21 only marginally affected the increase in thymidine incorporation by HGF (Fig. 1D, lane 4). This differential effect of p21 and p27 was specific for cells treated with HGF because ectopic expression of either of these cdk inhibitors in proliferating myoblasts promotes cell-cycle ar-

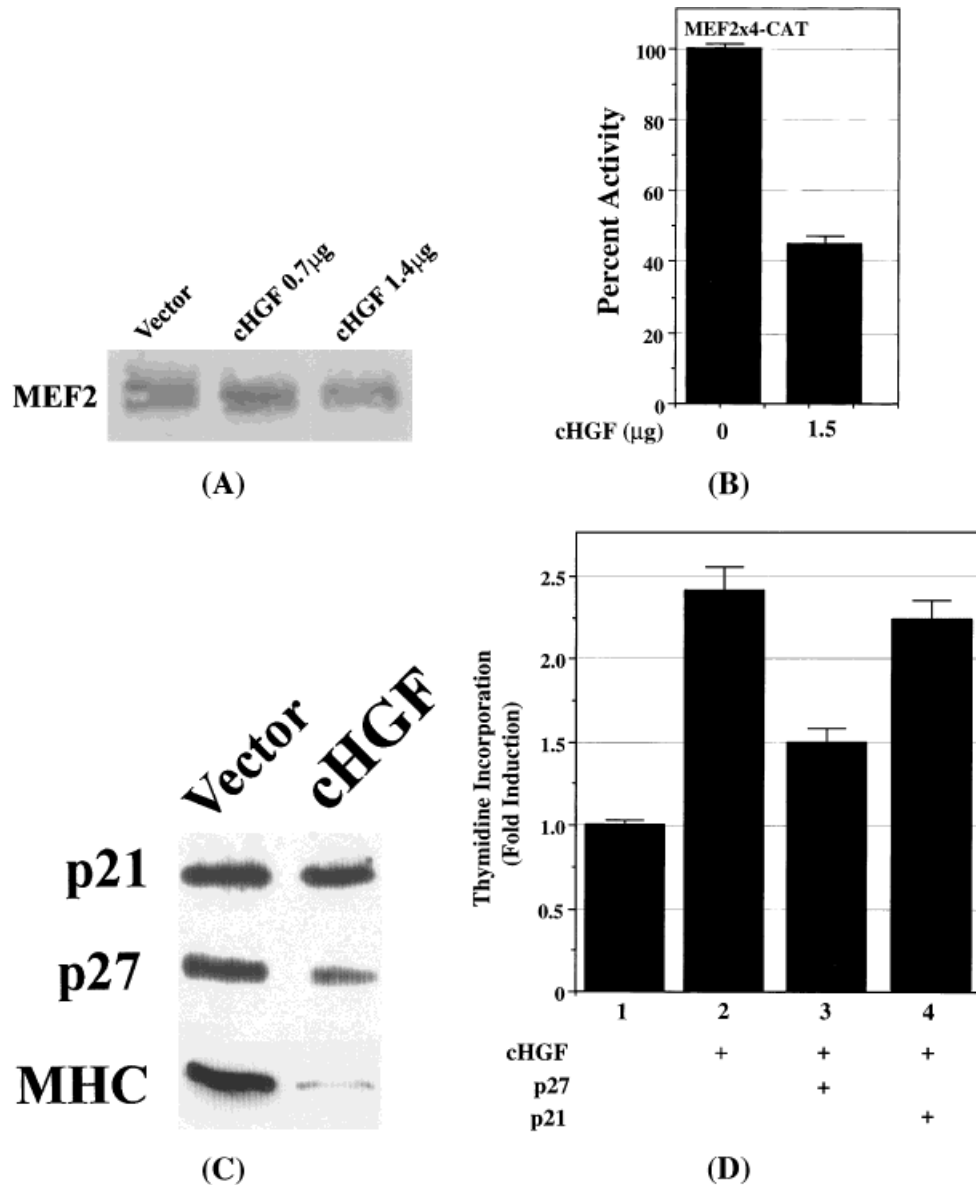


Fig. 1. Hepatocyte growth factor (HGF) affects MEF2 transactivation but not its expression. **A:** Western blot analysis of MEF2 proteins in chicken satellite cells transiently transfected with either cytomegalovirus (CMV)-cHGF or empty vector. **B:** Satellite cells were co-transfected with MEF2x4-chloramphenicol acetyl transferase (CAT; 0.2 µg) and CMV-β-gal (0.1 µg), and the indicated amounts of CMV-cHGF. Cultures were harvested 48 h post-transfection and CAT activity was determined and normalized to β-gal activity. Bars represent CAT activity relative to that of vehicle-transfected cells. Each value represents the mean and SEM of triplicate results. **C:** HGF

differentially affects cell regulatory proteins. Primary cultures of chicken satellite cells were transiently transfected with control vector or a construct encoding cHGF (2.0 µg). Cultures were harvested 48 h post-transfection and cell lysates were subjected to Western blot analysis of cell-cycle regulatory proteins. **D:** Ectopic p27 but not p21 can reverse HGF's effects on thymidine incorporation. Cells were transfected with CMV-cHGF (0.7 µg), with or without CMV-p21 (0.7 µg) or CMV-p27 (0.7 µg), and thymidine incorporation into DNA was analyzed two days post-transfection.

rest and differentiation in the absence of HGF (Skapek et al., 1995) (data not shown).

Twist expression is upregulated by HGF

The bHLH protein Twist has been previously shown to specifically inhibit MEF2 activity (Spicer et al., 1996). The possibility that HGF-dependent inhibition of MEF2 activity is due to changes in Twist expression

was examined. In an RT-PCR analysis of control and HGF-transfected satellite cells, Twist expression was markedly upregulated by HGF, relative to control cells that expressed only trace levels of the gene (Fig. 2A). Twist protein levels were similarly upregulated by HGF (Fig. 2B). This upregulation was accompanied by a reduction in the expression of MHC mRNA, as found previously (Gal-Levi et al., 1998).

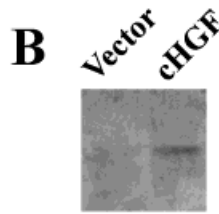
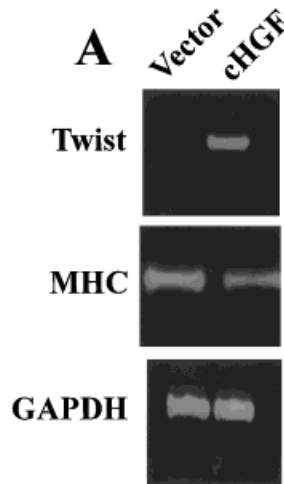


Fig. 2. Twist expression is upregulated by HGF. Chicken satellite cells were transiently transfected as described in Figure 1. Cultures were harvested and assayed for the expression levels of Twist, myosin heavy chain (MHC), and GAPDH by RT-PCR (A), or for Twist protein levels (B).

Similar effects of twist overexpression and HGF on myogenic differentiation markers

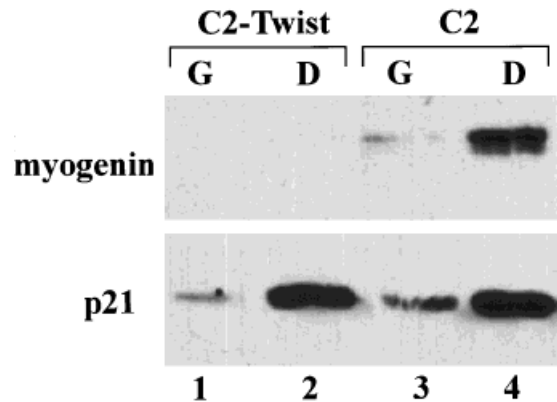
Transfection of satellite cells with expression constructs for HGF or chicken Twist resulted in a marked reduction in MHC expression, even at concentrations as low as 0.1 μ g of Twist-encoding construct (Fig. 3A). In mouse myogenic C2 cells, or in C2 cells stably expressing Twist, no myogenin protein was observed in the growing phase (Fig. 3B, lanes 1 and 3). However, in low-serum conditions, its expression was induced only in the parental cells and not in Twist-expressing cells (Fig. 3B, lanes 2 and 4). Similar results were obtained with the MHC expression (data not shown). In contrast, the induction of p21 was unaffected by the expression of Twist (Fig. 3B), as shown for HGF. Levels of p21 were the same as in the parental cells (data not shown).

p27 and Twist-AS block HGF-dependent inhibition of muscle differentiation

Satellite cells were co-transfected with HGF, with or without p27 and/or a construct containing chicken Twist cDNA subcloned in its reverse orientation (Twist-AS). Two days post-transfection, BrdU was added to the cells for an additional 4 h. Cells were then fixed and stained for BrdU incorporation as a measure of cells in the S phase, and for MHC expression by double-immunofluorescence (Fig. 4 and Table 1). More than 75% of the cells that were transfected with vector alone formed large myotubes expressing MHC while the rest were still cycling (Fig. 4, A–C, and Table 1). In contrast, approximately half of the cells transfected with HGF expressed BrdU and the other half expressed MHC (Fig. 4, D–F and Table 1). The morphology of the myotubes formed in the HGF-transfected cell population consisted of thin myotubes with few nuclei vs. large myotubes in the control cells (Fig. 4, compare A and C with D and F). Co-transfection of HGF with either Twist-AS (Fig. 4, G–I, and Table 1) or p27 (Fig.



(A)



(B)

Fig. 3. Similar effects of Twist overexpression and HGF on muscle-specific protein. **A:** Western blot analysis of MHC in satellite cells transfected with either empty vector, CMV-cHGF (0.7 μ g), or the indicated amount of CMV-Ctwist. **B:** C2 cells stably expressing Twist or parental cells were either grown in high-serum-containing medium (G) or induced to differentiate by switching to low-serum medium (D).

4, J–L, and Table I) partially rescued HGF's effect on the number of BrdU-incorporating and MHC-expressing cells compared to control cells. Only the combination of p27- and Twist-AS-encoding plasmids, even at half concentrations, in the presence of HGF completely abolished HGF's effects on BrdU and MHC expression (Table 1). Similar results for cell proliferation were seen in a dose-responsive manner up to 0.35 μ g of each construct (data not shown). Although both Twist-AS and p27 expression increased the number of myotubes seen in the presence of HGF, their morphology remained thin relative to control cells (Fig. 4, G and J). This morphology was only slightly recovered by combining p27 and Twist-AS (data not shown). Because it is a secreted protein, HGF affects a larger population of cells than would Twist-AS or p27, which act cell-autonomously, thus making their relative effects even more significant.

Coordinated regulation of Twist and p27

Next, we tested whether the rescue of differentiation by ectopic p27 expression is mediated by changes in Twist expression. Satellite cells were transfected with HGF in the presence or absence of p27 and assayed for Twist mRNA expression after two days. Ectopic p27 completely blocked the induction of Twist mRNA expression due to HGF (Fig. 5A), suggesting that reduction in p27 expression levels is required for the induction of Twist by HGF. In HGF-transfected cells, MHC and p27 protein levels were

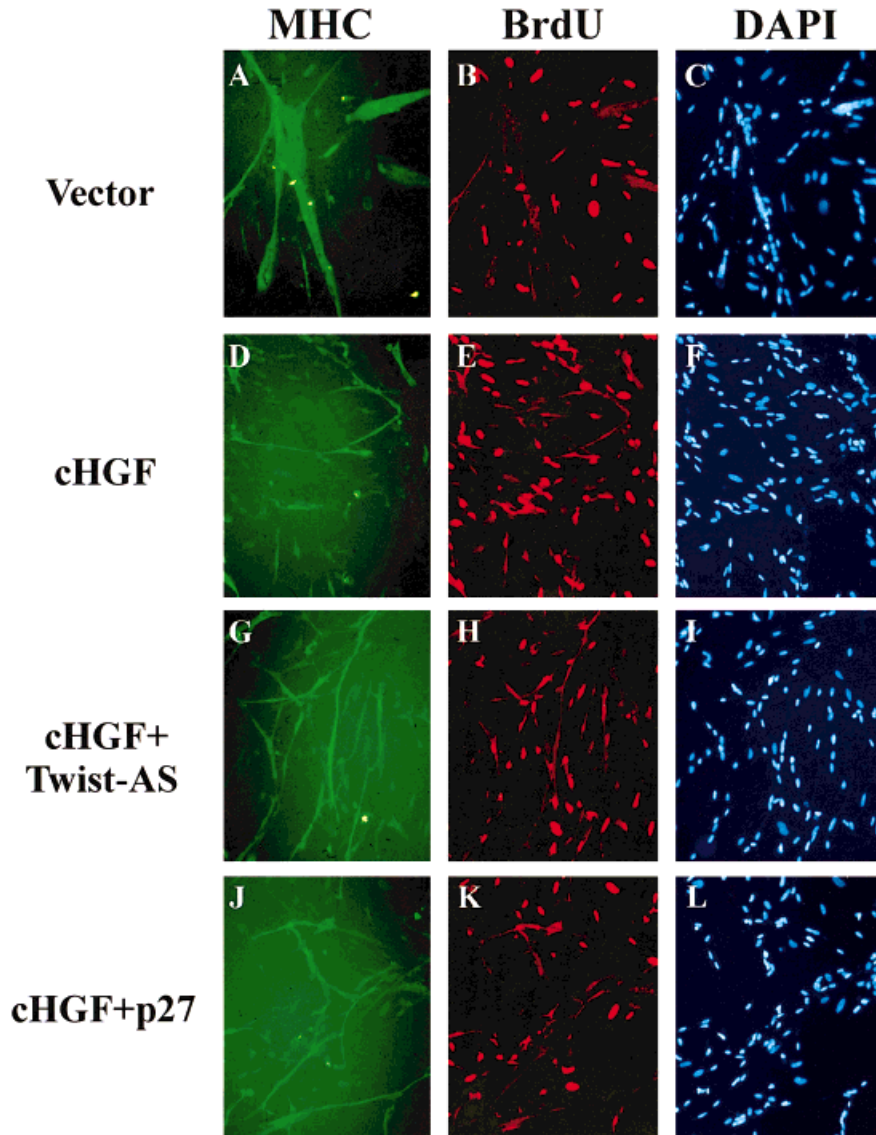


Fig. 4. Ectopic p27 and/or Twist-anti-sense (Twist-AS) reverse HGF inhibition of myogenic differentiation. Proliferating satellite cells were transfected with vector, CMV-cHGF (0.7 μ g) alone or with CMV-Ctwist-AS (1.4 μ g) or CMV-p27 (1.4 μ g) or a combination of the latter plasmids (0.7 μ g each). After 48 h, cells were fixed and immunostained for MHC (A, D, G, and J) and bromodeoxyuridine (BrdU) (B, E, H, and K). Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI) (C, F, I, and L).

TABLE 1. Distribution of MHC and BrdU staining in transfected cells¹

	% BrdU	% MHC	Total cell number
pCSA	24.3	75.7	2,053
cHGF	51.4	48.6	2,463
cHGF + Twist-AS	31.7	68.3	2,090
cHGF + p27	33.0	67.0	2,010
cHGF + p27 + Twist-AS	20.3	79.7	1,550

¹Data are the average of two independent experiments.

reduced compared to control cells (Fig. 5B; see Fig. 1C) in agreement with Gal-Levi et al. (1998). Co-expression of HGF and either Twist-AS or p27 restored MHC expression to levels close to control cells, whereas Twist-AS completely blocked the HGF-induced reduction in p27 levels, suggesting some degree of coordinate regulation between Twist and p27 levels during differentiation.

DISCUSSION

HGF has been shown to play a critical role in regulating myogenic differentiation during embryonic development as well as in adult satellite cells. It is required for the proliferation of fetal myoblasts (Maina et al., 1996) and has the unique property of being able to activate, induce the proliferation of, and inhibit the differentiation of adult myoblasts and satellite cells (Allen et al., 1995; Gal-Levi et al., 1998; Miller et al., 2000). The molecular mechanism via which HGF delays withdrawal from the cell cycle and prevents differentiation in satellite cells was studied. We suggest that HGF's effects are specifically mediated through the bHLH protein Twist and the cdk inhibitor p27. First, HGF markedly induced the expression of Twist while decreasing the level of p27. Second, ectopic expression of p27 or a construct expressing Twist cDNA in the antisense orientation (Twist-AS) each partially overcame the effects of HGF, while their combination

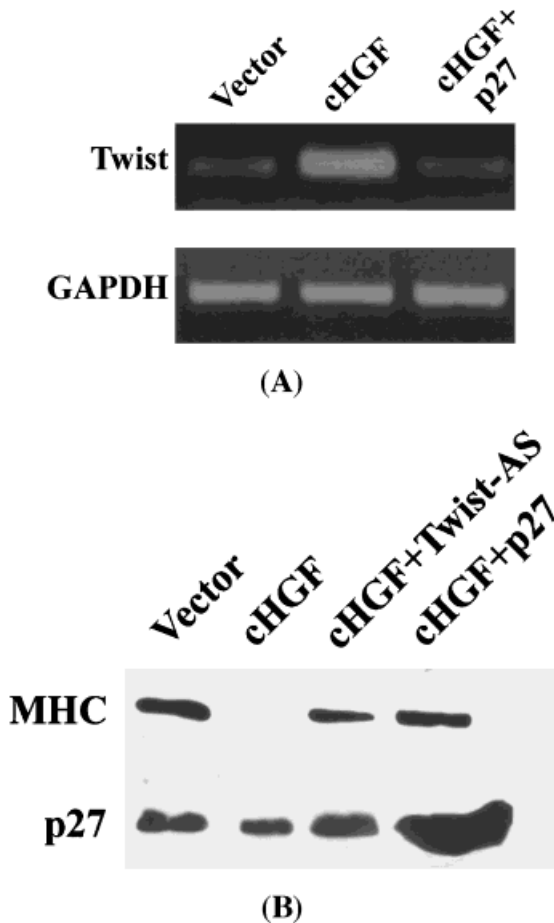


Fig. 5. **A:** p27 expression blocks the induction of Twist by HGF. RT-PCR analysis for Twist and GAPDH expression in satellite cells transfected with either empty vector or CMV-cHGF (0.7 μ g) with or without CMV-p27 (1.4 μ g). **B:** Proliferating satellite cells were transfected with vector, CMV-cHGF (0.7 μ g) alone or with CMV-Ctwist-AS (1.4 μ g) or CMV-p27 (1.4 μ g). After 48 h, cells were analyzed for protein expression by Western blot analysis

completely blocked the HGF-induced increase in cell proliferation and inhibition of differentiation.

Twist has been shown to be expressed and regulated mainly during embryonic development in *Drosophila* (Bate et al., 1991) as well as in vertebrates (Hopwood et al., 1989; Wolf et al., 1991; Fuchtbauer, 1995; Stoetzel et al., 1995; Gitelman, 1997). In this study, we show for the first time that Twist plays a role in the processes of adult muscle growth and regeneration. Both HGF and Twist inhibited muscle differentiation proteins without any effect on p21 expression in chicken satellite cells or in mouse myogenic cells. This phenomenon has been seen in other myogenic systems. For instance, MyoD^{-/-} myogenic cells poorly differentiated in culture, and this was accompanied with delayed induction of myogenin and no induction of desmin; however, no effect on p21 upregulation was observed (Sabourin et al., 1999). Twist has been recently shown to antagonize p53 without affecting p21 expression during p53-dependent apoptosis (Maestro et al., 1999). Interestingly, overexpression of HGF reduced p53 expression in chicken satellite cells (data not shown). Taken to-

gether, these results implicate Twist as a mediator of HGF action in adult myoblast proliferation and differentiation. We believe that Twist is upregulated by HGF via a direct mechanism and not only as a result of the cell failure to undergo growth arrest, as we have found that insulin-like growth factor I behaved as a mitogen for chicken satellite cells but did not affect Twist or p27 expression levels (Duclos et al., 1991; Hodik et al., 1997) (data not shown).

What is the possible mechanism by which HGF regulates proliferation and differentiation of muscle cells? HGF induced Twist and reduced p27 levels. The reduction in p27 levels is of significance because ectopic p27 was able to override inhibition of differentiation by HGF (Fig. 3). Moreover, it appears that the reduction in p27 is required for Twist induction by HGF (Fig. 5). Thus, both the induction of Twist by HGF and the reduction of p27 to threshold levels are required for the inhibitory activity of HGF on muscle cell differentiation. Our notion is that HGF independently regulates these two factors since only the combination of p27 and Twist-AS could completely block HGF's effects on proliferation and differentiation. Still, it is tempting to speculate that Twist may somehow regulate p27 levels because inhibition of Twist induction by HGF allowed p27 to increase to normal levels and restore MHC (Fig. 5). Taken together, we suggest that p27 and Twist are coordinately regulated and, as satellite cells proliferate in response to HGF, p27 levels slowly increase to a threshold level, at which time the cells exit the cell cycle, turn off Twist expression, and undergo differentiation.

Of the two cdk inhibitors tested, p27, but not p21, was downregulated by HGF, and ectopic p21 could not override the effect of HGF on cell proliferation (Fig. 1). This is consistent with the proposed role of p27 as a trigger for differentiation. Durand et al. (1997) have shown that small changes in the level of p27 play a crucial role in determining the timing of differentiation. During the proliferation of oligodendrocyte precursor cells, p27 levels slowly accumulate, consistent with a role as a cell-intrinsic timer which arrests the cells and promotes their differentiation only after reaching a threshold level (Durand et al., 1997, 1998). At present, it is unclear whether, in adult muscle, p27 inhibits Twist expression by causing growth arrest or whether a more direct mechanism exists. However, it is worth noting that during embryonic development, Twist does not seem to be expressed in post-mitotic cells (Hopwood et al., 1989; Wolf et al., 1991; Fuchtbauer, 1995; Stoetzel et al., 1995; Gitelman, 1997).

The morphology observed in the presence of ectopic HGF suggests that HGF, in addition to its effects on cell-cycle exit and muscle-specific gene expression, also affects very late events, such as myotube fusion. The morphology of the cells was not restored by ectopic p27 or Twist-AS, suggesting that other molecules mediate the effects of HGF on myoblast fusion. HGF has been shown to interfere with N-cadherin and integrin-matrix interactions in epithelial cells, thereby disrupting cell-cell contact and disorganizing epithelial architecture (Peluso, 1997; Alford et al., 1998). These proteins, as well as other cell-surface receptors, play an important role in the fusion of terminally differentiated myoblasts (Knudsen et al., 1990; Donalies et al., 1991;

Hynes, 1992; Eng et al., 1997; Redfield et al., 1997) and, as such, may be involved in HGF inhibition of cell fusion.

In summary, our findings suggest that HGF coordinately regulates the expression of p27 and Twist to mediate its effects on adult muscle cell proliferation and differentiation. The induction of Twist by HGF enables these myogenic cells to proliferate and not differentiate until sufficient cell numbers have been produced. Furthermore, HGF's effects on muscle cell morphology and fusion most probably require additional mediators.

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