CELL STRUCTURE AND FUNCTION 27: 21–27 (2002)

Possible Involvement of Hic-5, a Focal Adhesion Protein, in the Differentiation of C2C12 Myoblasts

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ABSTRACT. **Hic-5, a focal adhesion protein, has been implicated in cellular senescence and differentiation. In this study, we examined its involvement in myogenic differentiation. The *hic-5* expression level in growing C2C12 myoblasts increased slightly on the first day and then gradually decreased until no *hic-5* was detectable after 7 days of differentiation. *In vivo*, its expression level declined in the thigh and the calf skeletal muscle of mouse embryos after birth. The introduction of an antisense expression vector of *hic-5* into C2C12 cells decreased the number of clones expressing the myosin heavy chain (MHC) upon exposure to the differentiation medium. In the cloned cells with low levels of *hic-5,* the efficiency of myotube formation was significantly reduced. The expression levels of MyoD, myogenin, MHC and p21 were also reduced in these clones. The results suggested that *hic-5* plays a role in the initial stage of myogenic differentiation.**

Key words: Hic-5/C2C12/myogenic differentiation

Although lineage-specific transcription factors like MyoD, Myf5, myogenin and MEF2 play central roles in the differentiation of cells of the myogenic lineage (Arnold and Winter, 1998), there is a body of evidence that points to the importance of the cellular microenvironment, representative of which are cell-extracellular matrix (ECM) interactions during embryonic development and cellular differentiation, including myogenesis (Adams and Watt, 1993; Gumbier, 1996). For example, laminin-1 plays a prominent role in promoting myotube formation along with adhesion and proliferation of myoblasts, while fibronectin inhibits myogenic differentiation (von der Mark and Ocalan, 1989). Integrins at the cell surface are thought to mediate the signals of the cell-extracellular matrix interactions, regulating development and cellular differentiation as well as cell shape, motility, and growth (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Fassler *et al*., 1996). Menko and Boettiger demonstrated that the interaction of integrin with ECM was essential to initiate the terminal stages of myogenic differentiation (Menko and Boettiger, 1987).

hic-5 was first isolated as TGFβ1- and hydrogen peroxide-inducible cDNA from mouse osteoblastic cells (Shibanuma *et al*., 1994). Structurally, it possesses four LIM and three LD domains, both of which potentially serve as interfaces for protein-protein interactions, in its C- and N-terminal regions, respectively, and shows striking similarity to paxillin (Turner, 2000; Thomas *et al*., 1999). Its localization at focal adhesions and structural features suggested that Hic-5 plays some role as an adaptor molecule in integrin signaling through interaction with several kinds of signaling molecules (Nishiya *et al*., 2001), thereby modulating cell phenotypes. In fact it has been implicated in the cellular senescence of fibroblasts and differentiation of osteoblasts (Shibanuma *et al*., 1997; Shibanuma and Nose, 1998; Ishino *et al*., 2000). Its association with platelet development was also reported by Hagmann *et al*. (Hagmann *et al*., 1998), although the molecular mechanisms underlying its biological effects are still largely unknown.

The molecular aspects and biological effects of Hic-5 as described above suggested its involvement in broad range of phenotypic change of cells that were associated with integrin signal. We here examined its involvement in the differentiation of C2C12 myoblasts, where cell-ECM interactions and/or integrin signals are supposed to play important roles in modulating the phenotypes as mentioned above.

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Abbreviations: MHC, myosin heavy chain; MLC, myosin light chain; ECM, extracellular matrix; GD, glyceraldehyde-3-phosphate dehydrogenase.

Materials and Methods

Cell culture

Mouse myoblastic cells, C2C12, were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 15% fetal bovine serum and 50 µg/ml kanamycin under a humidified atmosphere of 5% CO_2 in air. To induce the differentiation, the medium was replaced with the differentiation medium containing 2% horse serum instead of 15% fetal bovine serum.

Northern blots

Total RNA was extracted, purified and Northern blotted essentially as described in Shibanuma *et al*., 1997. The probes for *hic-5* and glyceraldehyde-3-phosphate dehydrogenase (GD) were the same as in Shibanuma *et al*., 1997. Mouse cDNA fragments of MyoD, myogenin, myosin heavy chain (MHC) and myosin light chain (MLC) provided by Dr. A. Asakura (Fred Hutchinson Cancer Research Center, Seattle) and of p21 provided by Dr. B. Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MA) were used as probes. The radioactivity was quantified with a Bioimage Analyzer (Fuji Photo Film, Tokyo). The values were normalized to that of GD as a monitor of the amount of RNA in each lane.

Expression plasmids, transfection and analysis of clones

Expression vectors, CMV/S5 (Shibanuma *et al*., 1997) for sense and pEF-BOS-anti-*hic-5* (Shibanuma and Nose, 1998) for antisense expression of *hic-5*, were introduced into C2C12 cells together with pSV2neo by the conventional calcium phosphate coprecipitation method (Graham and van der Eb, 1973), and the cells were selected with G418 (400 µg/ml) for 10 days. As a control plasmid, the empty vector, pRc/CMV or pEF-BOS, was used for transfection. The colonies were then placed in the differentiation medium for 8 to 12 days to analyze MHC expression or picked up and expanded for clonal analysis. Western blotting was performed as described previously (Ishino *et al*., 2000).

Immunostaining for MHC

The colonies were first treated with 0.1% saponin for 15 sec, fixed with 3.7% formalin for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min as previously reported (Arber *et al*., 1994). Subsequent processes were done as described previously (Ishino *et al*., 2000). The first antibody used was monoclonal anti-skeletal myosin M-4276 (Sigma Chemical Co., St. Louis, MO), and the second antibody was anti-mouse IgG peroxidase-labeled (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD). The immunocomplex was visualized with a DAB Substrate Kit (Vector Laboratories, Inc., Burlingame, CA).

Mouse embryos

Pregnant ICR mice at 12 days of gestation were purchased from the Saitama Experimental Animals, Co., Saitama.

Results and Discussion

We first examined expression patterns of *hic-5* during myogenic differentiation *in vitro*. Logarithmically growing C2C12 cells were exposed to a low mitogen medium containing 2% horse serum to cause them to differentiate. After a given period, total RNA was extracted and Northern blotted with probes for *hic-5* along with those for MyoD, myogenin, myosin heavy chain (MHC) and myosin light chain (MLC). MyoD and myogenin are basic helix-loop-helix transcription factors controlling skeletal muscle development at the initial stage (Arnold and Winter, 1998). MHC and MLC are proteins expressed typically in differentiated skeletal muscle cells and used as differentiation markers of the myogenic lineage. Figure 1 shows that the expression level of *hic-5*, among the above mRNAs, was the first to change; a high level of expression was detected in the proliferating myoblasts and the expression increased slightly on the first day after the switch. It then decreased and became hardly visible after 7 days. Changes of MyoD and myogenin expression followed those of *hic-5* expression; their levels increased with a peak on the second day and then decreased. Unlike these transcripts, MHC and MLC, which were undetectable early in the process, began to be expressed after 2 days, followed by the formation of myotubes. Myotubes were visible 6 days after the switch (data not shown).

A similar change in the expression pattern of *hic-5* was observed *in vivo* during development of the skeletal muscle. As shown in Fig. 2, *hic-5* mRNA was highly expressed in the thigh and the calf muscle from embryonic day 16 to 18. After birth, its expression decreased and became barely detectable in the adult muscle. From these expression patterns, *hic-5* was speculated to play some role in the regulation of the terminal differentiation of myoblasts at an early stage in the process.

To test the above possibility directly, we transfected sense (CMV/S5) and antisense (pEF-BOS-anti-*hic-5*) expression vectors of *hic-5* carrying a neomycin resistance gene into C2C12 cells and assessed the effects of their expression on the C2C12 differentiation (Fig. 3). Following selection with G418 for 10 days, colonies were placed in the differentiation medium with low serum for 8 or 12 days. The density of the colonies was controlled at about 50 to 100 colonies/ 90 mm dish, to prevent the enhancement of myotube formation due to cell-cell interactions. As a differentiation marker, the expression of MHC was evaluated by immunostaining, and colonies positively stained with the antibody to MHC were enumerated. The sense expression affected marginally the number of MHC-positive colonies (Fig. 3A). The overexpression of *hic-5*, thus, might lead to neither enhancement nor suppression of MHC expression. Alternatively, the cells that overexpressed *hic-5* would selectively die of apoptosis, making it impossible to evaluate the effects of overexpressing *hic-5* in this kind of assay. Taken the re-

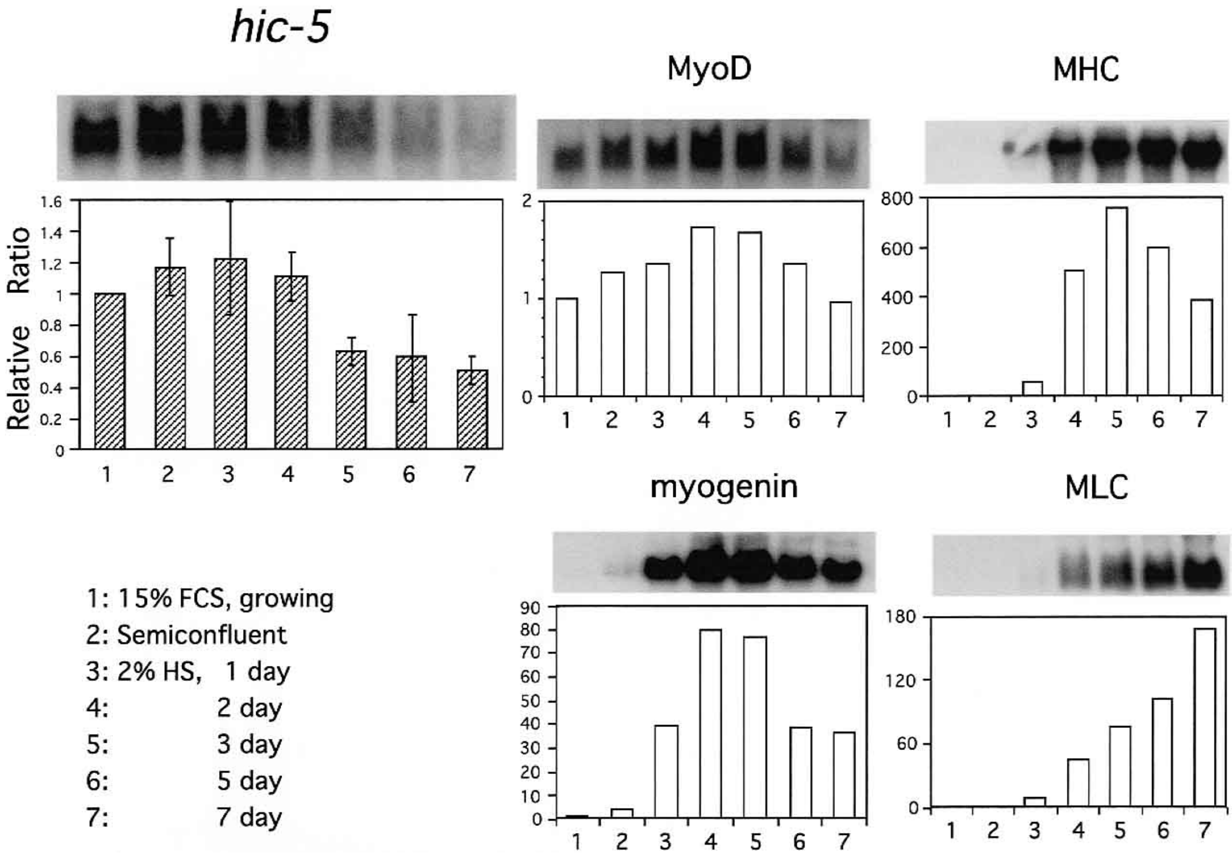


Fig. 1. Expression pattern of *hic-5* during the myogenic differentiation of C2C12 cells. Total RNA was extracted from C2C12 cells while growing (lane 1), at semiconfluence (lane 2), or placed in the differentiation medium for 1 (lane 3), 2 (lane 4), 3 (lane 5), 5 (lane 6) or 7 (lane 7) days, and Northern blotted with the probes indicated. The mRNA level was normalized to that of GD and is shown as a ratio to that in growing cells.

cent report by Hu *et al*. showing that overexpression of *hic-5* resulted in the inhibition of myogenesis by inducing apoptosis (Hu *et al*., 1999), we favored the latter possibility. Consistent with this idea, no clones expressing *hic-5* over 2-fold to the parental cells were obtained after screening more than 50 clones in our trial to establish a stable cell line overexpressing *hic-5*. In contrast to the sense expression, the antisense expression evidently reduced the number of colonies expressing MHC (Fig. 3B). This observation suggested that expression of *hic-5* above a certain level was required for efficient induction of MHC.

The involvement of *hic-5* in the myogenic differentiation was further examined in detail by isolating and analyzing each of the antisense-transfected colonies; the G418-resistant colonies obtained in the antisense-transfected populations as above were isolated, expanded, and induced to differentiate. As shown in Fig. 4A, the *hic-5* protein levels of the clones varied from 0.63 to 1.1 of that in the parental C2C12 population. Although the difference in the level of *hic-5* expression among the clones was modest, the *hic-5* expression level was well correlated with the efficiency of myotube formation among 6 independent clones examined; as shown in Fig. 4B, the clones expressing almost the same level of Hic-5 as parental cells, such as #5 and #9, had well-developed myotubes and so did to a lesser extent, clone #10, being consistent with their *hic-5* expression level of 1.0, 1.1, and 0.85, respectively. However, the myotube formation was significantly impaired in clones such as #22 and #B4 whose *hic-5* expression levels were reduced to 0.63 or 0.73. Consistent with this, MHC mRNA expression was also remarkably suppressed in these clones (Fig. 5A).

The core regulatory network for the myogenic differentiation has been identified at a molecular level, and a genetic hierarchy has been revealed among the molecules controlling skeletal muscle development (Arnold and Winter, 1998); MyoD or Myf5 specifies cells to adopt the myoblast fate and then, in response to a trigger of the differentiation, the expression of myogenin and p21, which are required to control the differentiation process directly and cell-cycle arrest (Parker *et al*., 1995; Zhang *et al*., 1999), respectively, is induced. Following these molecular events, muscle-specific proteins are produced, such as MHC and MLC. On the basis of this model, we examined the mRNA levels of MyoD and myogenin among the above clones and tried to obtain some

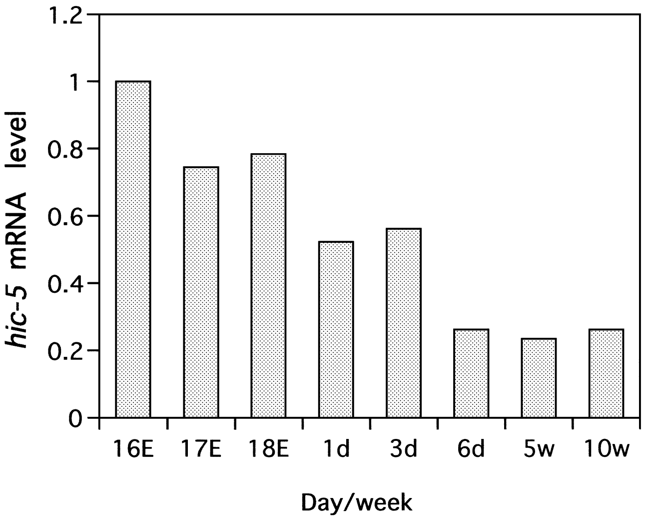


Fig. 2. Expression pattern of *hic-5* in developing skeletal muscle. Total RNA was extracted from the thigh and the calf of an embryo or postnatal mouse of the indicated age (ICR strain), and Northern blotted with the *hic-5* probe. The mRNA level was normalized to that of GD and indicated as a ratio to that at embryonic day 16. E, d, and w stand for embryonic, postnatal day and week, respectively.

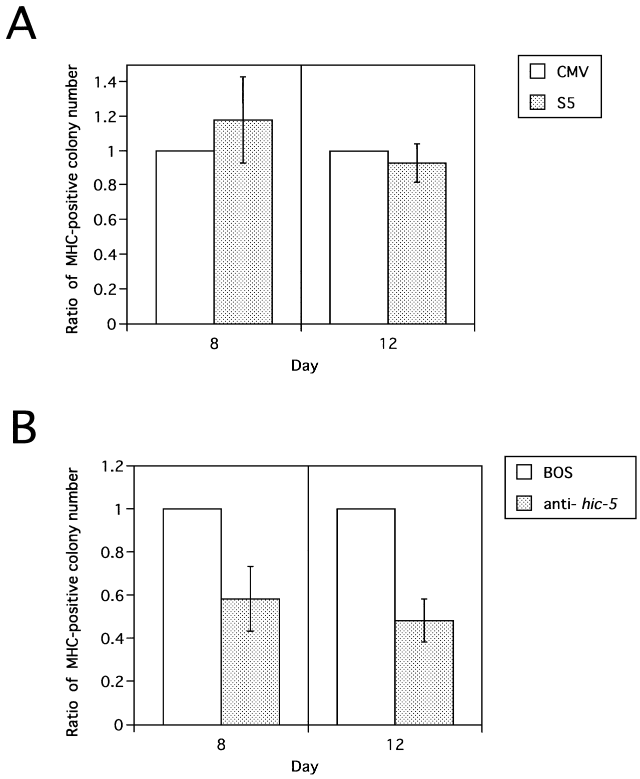


Fig. 3. Effects of sense and anti-sense expression of *hic-5* on the efficiency of MHC expression in differentiating C2C12 cells. CMV/S5 (A, black bar) or pEF-BOS-anti-*hic-5* (B, black bar) was introduced into C2C12 cells along with pSV2neo, and G418-resistant cells were grown for 10 days. pRc/CMV (A, white bar) or pEF-BOS (B, white bar) was used as a control plasmid. Colonies on the dishes were then placed in the differentiation medium for 8 or 12 days and immunostained with the antibody to MHC. The number of colonies positive for MHC was counted and normalized to the total number of colonies on the dish. The total number of colonies was around 100 in each experiment, 60% of which were MHC-positive in the control experiment. The experiments were repeated at least three times. The values are means $\pm$ SD of the ratios to the control.

insight into the stage of differentiation at which *hic-5* was involved. In clones #B4 and #22 whose expression levels of *hic-5* were relatively low, the expression of myogenin as well as MHC was significantly repressed (Fig. 5A). MyoD expression levels were also reduced in these clones, though to a lesser extent, upon exposure to the differentiation medium (Fig. 5A, lanes 2 to 4 labeled by MyoD); although in the proliferating state, the average level of MyoD expression in the low-expressing clones was comparable (0.83) to that in the parental cells (Fig. 5A, lane 1 labeled by MyoD), the level was reduced to 0.44 of that in the parental cells in the differentiating state. To determine whether the decrease in MyoD in the differentiating #B4 and #22 cells was biologically significant or not, we examined p21 expression levels in the clones. According to a previous report, MyoD was a positive regulator of the up-regulation of p21 (Guo *et al.*, 1995; Halevy *et al.*, 1995). In the present experiments, the induction of p21 expression was found to be attenuated in #B4 and #22, compared to that in the other clones expressing *hic-5* at the level comparable to the parental cells, as expected (Fig. 5). These results suggested that *hic-5* was primarily required for the expression of MyoD and myogenin at the initial stage of the differentiation shortly after cell commitment, maintaining the potential of myoblasts to differentiate and/or preventing them from apoptosis. The clones studied here all exhibited low apoptotic index in the differentiation condition, which hindered our analysis of the effects of Hic-5 on apoptosis (data not shown). In conclusion, it was suggested that low-level expression of *hic-5* below a critical threshold resulted in the blockage of a cascade of the molecular events subsequent to the MyoD and myogenin expression. The expression pattern of *hic-5* described above was consistent with this hypothesis.

In the present study, we demonstrated that *hic-5* was one of the positive regulators in myogenic differentiation. This conclusion is in contrast with a previous report by Hu *et al.* that *hic-5* blocked the differentiation of C2C12 cells (Hu *et al.*, 1999). This apparent contradiction would be accounted for by the difference in strategy to elucidate the *hic-5* function; they overexpressed *hic-5* in the cells and observed that the *hic-5*-overexpressing clones were incapable of forming myotubes and instead died of apoptosis. We employed an antisense expression system to avoid the non-physiological response inevitable to ectopic overexpression of a protein, and rather reduced the expression level of *hic-5*. Recently, we have demonstrated the biological significance of *hic-5* function in integrin signaling and characterized the mode of action of *hic-5* as a competitor of paxillin, another adaptor

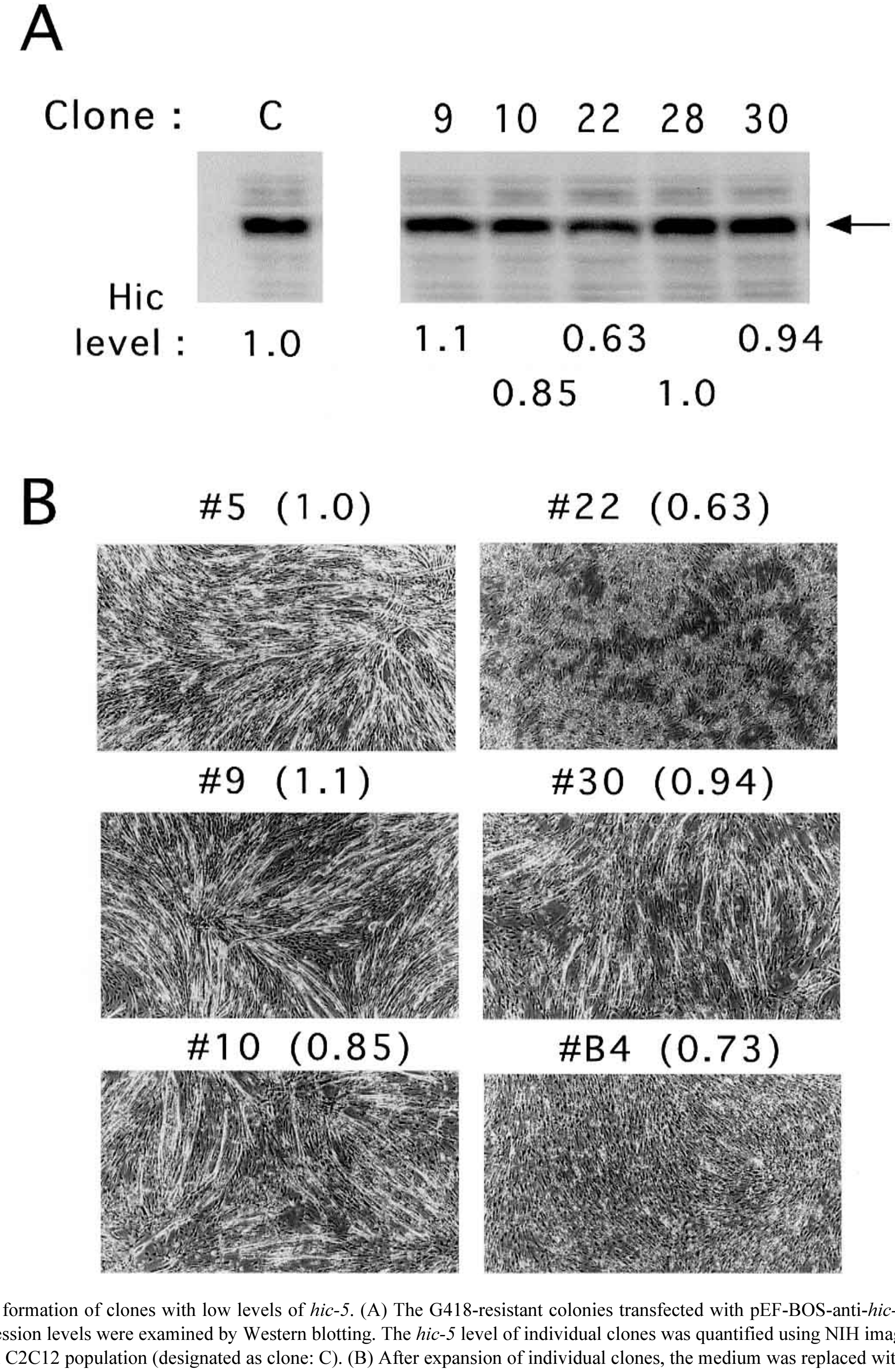
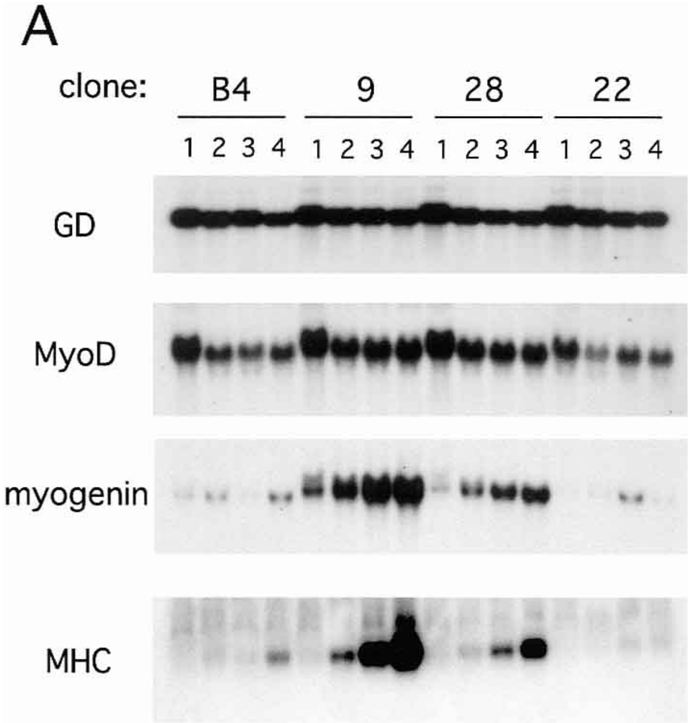


Fig. 4. Myotube formation of clones with low levels of *hic-5*. (A) The G418-resistant colonies transfected with pEF-BOS-anti-*hic-5* were isolated, and *hic-5* protein expression levels were examined by Western blotting. The *hic-5* level of individual clones was quantified using NIH image and normalized to that of the parental C2C12 population (designated as clone: C). (B) After expansion of individual clones, the medium was replaced with differentiation medium, and photographs were taken after 10 days. The value in parentheses is the *hic-5* protein expression level of the clone, which was examined in (A).

molecule involved in the integrin signaling (Turner, 2000; Nishiya *et al*., 2001). Thus, a small change in the amount of *hic-5*, either an increase or decrease, potentially causes a shift in the balance of the relative concentrations of *hic-5* and paxillin, leading to quantitative and qualitative changes in integrin signaling. The profound effects of *hic-5* expres-



B

clone	Hic-5 level	p21 (fold induction)
C2C12	1.0	3.1
5	1.0	5.1
9	1.1	5.7
10	0.85	1.4
22	0.63	0.94
28	1.0	5.5
B4	0.73	1.9

Fig. 5. Expression levels of MyoD, myogenin and MHC in clones with low levels of *hic-5*. (A) Total RNA was extracted from clones in a semiconfluent state (lane 1), or 1 (lane 2), 2 (lane 3) and 3 (lane 4) days after the switch to differentiation medium, and Northern blotted with the probes indicated. Quantitative analysis using NIH image showed that the average of the ratio of MyoD expression level in the low expressing clones (#B4 and #22) to the high expressing clones (#9, #28, and #10) was 0.83 before (lane 1) and 0.44 after the switch (lanes 2 to 4). (B) RNA extraction and Northern blotting were performed as in (A) using p21 probe. The intensity of the bands on X-ray film was quantified with NIH image, normalized to that of GD, and the fold-induction at 1 day after the switch is shown with the level of *hic-5* protein in the individual clones.

sion on myogenic differentiation, evident from the antisense expression observed here and the overexpression in a previous report (Hu *et al*., 1999), could be the consequences of such an alteration of integrin signaling. Before reaching a conclusion, further experiments will be needed that elucidate the role of *hic-5* at physiological conditions.

An intricate network controlling the process of morphogenesis and cell differentiation is composed of molecular machinery operating in signaling cascades, transcription complexes, cellular microenvironment and so on. It is thought that one of the functions of cell-ECM interactions or the cellular microenvironment in the above process is to maintain differentiated phenotypes of a variety of cells (Adams and Watt, 1993). Although ECM or its receptor, integrin, has been shown to be part of the above network, little is known about how it regulates the process. Additional study of the molecular basis of *hic-5* functions will shed light on these issues. It would also provide a molecular tool to manipulate the fate of cells of mesenchymal origin such as fibroblasts, osteoblasts and myoblasts, whose phenotypes are modulated by *hic-5* in common as demonstrated by others (Shibanuma *et al*., 1997; Shibanuma and Nose, 1998; Ishino *et al*., 2000; Hu *et al*., 1999) and the present study.

Acknowledgments. This work was supported in part by Grants-in-Aid for Scientific Research, a Grant-in-Aid for Cancer Research, and High-Technology Research Center Project from the ministry for Education, Science, Sports, and Culture of Japan, and a Grant-in-Aid from the Takeda Science Foundation, Osaka. We thank Dr. A. Asakura (Fred Hutchinson Cancer Research Center, Seattle) for providing the cDNA of myogenic markers and Dr. B. Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MA) for that of mouse p21.

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(*Received for publication, October 25, 2001*
and in revised form, December 13, 2001)