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Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation

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Abstract

The role of hepatocyte growth factor (HGF) and its receptor, c-met, in proliferation and differentiation of satellite cells was studied in primary cultures of chicken skeletal muscle satellite cells and a myogenic C2 cell line. HGF mRNA was expressed mainly in the myotubes of both cultures. The addition of conditioned medium derived from those cultures had a scattering effect on the canine kidney epithelial cell line, MDCK. In contrast, c-met mRNA levels decreased during cell differentiation of C2 and primary satellite cells. Application of exogenous HGF to chicken myoblasts resulted in their enhanced DNA synthesis. Among several growth factors, HGF was the first to induce DNA synthesis in quiescent satellite cells, thereby driving them into the cell cycle. Ectopic expression of chicken HGF in primary satellite cells suppressed the activation of muscle-regulatory gene reporter constructs MCK-CAT, MRF4-CAT, MEF2-CAT and 4Rtk-CAT, as well as the gene expression of MyoD and myogenin, and MHC protein expression. Ectopic MyoD reversed HGF's inhibitory effect on MCK transactivation. These data suggest that HGF inhibits cell differentiation by inhibiting the activity of basic helix-loop-helix (bHLH)/E protein heterodimers, thus inhibiting myogenic determination factor activity and subsequent muscle-specific protein expression. During muscle growth and regeneration, HGF plays a dual role in satellite-cell myogenesis, affecting both the proliferation and differentiation of these cells in a paracrine fashion. © 1998 Elsevier Science B.V.

Keywords: Hepatocyte growth factor; Satellite cell; Differentiation

1. Introduction

Myogenic differentiation entails a cascade of intracellular events involving the coordination of muscle-specific gene expression and withdrawal from the cell cycle, and resulting in terminally differentiated myotubes. Previous studies have demonstrated the MyoD family of basic helix-loop-helix (bHLH) transcription factors (MyoD, myf5, myogenin, MRF4), known as myogenic determination factors (MDFs). These factors form heterodimers with ubiquitous bHLH nuclear proteins (E proteins) and act in cooperation with the MEF2 family members, thereby binding E boxes (MEF1) and MEF2 sites on muscle-specific gene promoters and activating these genes (reviewed in [1–3]).

The ability of adult muscle tissue to grow or regenerate in response to injury is solely dependent

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on the activation and proliferation of satellite cells. These small mononucleated cells first identified by Mauro [4] lie under the basal lamina of the myofiber, and are uniformly distributed throughout the length of the muscle (reviewed in [5]). Satellite cells are present in the muscle mostly in their quiescent state. Nevertheless, upon muscle injury, satellite cells become mitotically active, displaying programmed proliferation and differentiation followed by expression of muscle-specific proteins (reviewed in [6]). The extracellular factors that activate quiescent satellite cells and regulate their proliferation and differentiation processes during muscle growth and regeneration have not been completely elucidated. Nevertheless, several growth factors have been implicated in stimulating or inhibiting satellite-cell proliferation and differentiation. These factors include basic and acidic FGF (bFGF and aFGF), insulin-like growth factor I and II (IGF-I and IGF-II), TGF beta (TGF-β; reviewed in [7,8]), PDGF [9], and growth hormone [10].

HGF is a multifunctional cytokine that has pleiotropic effects on several cells and tissues. It was independently identified as a scatter factor (SF) which increased the motility of several normal and neoplastic cells [11], and the identity of this scatter factor as HGF was then unequivocally confirmed [12,13]. HGF is presumed to play an important role in activating hepatocytes during liver regeneration and to be involved in the proliferation of renal tubule cells, and endothelial and epithelial cell lines (reviewed in [14]). The HGF receptor is a protein encoded by the proto-oncogene c-met, a 190 kDa transmembrane tyrosine kinase [13,15].

Jennische et al. [16], who detected HGF mRNA in regenerating skeletal muscle, were the first to associate HGF with skeletal muscle. In addition, HGF and c-met have been shown to be expressed in developing limb buds by in-situ hybridization [17–19]. Transgenic mice overexpressing HGF exhibited ectopic development of skeletal muscle, indicating that HGF possesses physiological scatter activity in vivo in skeletal muscle development [20]. Moreover, Bladt et al. [21] showed that in c-met null mutant mouse embryos, the limb bud is not occupied by myogenic precursor cells, thus skeletal muscle is not formed. In addition, reducing the ability of c-met to transduce its signal in vivo caused a striking reduction in limb muscles, coupled to a generalized deficit of secondary fibers [22]. Recently, HGF has been shown to activate rat quiescent satellite cells and drive them into the cell cycle [23], and to be involved in their myogenesis [24].

To further elucidate the effect of HGF on proliferation and its molecular role in the differentiation process of satellite cells which are important for skeletal muscle regeneration, we employed primary cultures of chicken satellite cells, as well as a myogenic C2 cell line derived from mouse satellite cells [25]. Our data suggest that during muscle growth and regeneration, HGF plays a dual role in satellite-cell myogenesis: on one hand it induces quiescent cells to enter the cell cycle and enhances their proliferation. On the other, it inhibits cell differentiation, by inhibiting the activity of bHLH/E protein heterodimers, thus inhibiting MDF activity and subsequent muscle-specific protein expression.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (St. Louis, MO, USA). Sera and antibiotic–antimycotic solution were purchased from Bio-Lab (Jerusalem, Israel). dATP (α-^{32}P-labeled, 3000 Ci/mmol), [^{3}H]acetyl-CoA (200 Ci/mmol) and [^{3}H]thymidine (70 Ci/mmol) were obtained from DuPont NEN (Boston, MA, USA). Chicken embryo extract and lipofectamine were obtained from GibcoBRL (Gaithersburg, MD, USA). Recombinant human HGF was purchased from R & D Systems (Minneapolis, MN, USA). Recombinant human bFGF was a gift from Dr. G. Neufeld (Technion, Haifa, Israel). Chicken IGF-I was obtained from Genzyme (Boston, MA, USA).

2.2. Plasmids

The pCSAcHGF construct was made by subcloning a 3.2 kb *Cla*I–*Sma*I fragment containing full-length chicken HGF (pCHD) cloned by Thery et al. [19] into *Eco*RV–*Cla*I sites of pCSA plasmid

containing the cytomegalovirus promoter [26]. −3300MCK-CAT is a chloramphenicol acetyl transferase (CAT) reporter gene driven by skeletal muscle creatine kinase promoter (a gift from S. Hauschka, University of Washington, Seattle, WA, USA) described previously [27]. −336MRF4-CAT [28] was a gift from S. Konieczny (Purdue University, West Lafayette, IN, USA). MEF2-CAT, CAT reporter plasmid driven by four reiterated MEF2 sites upstream to the minimal Xenopus MyoD1a promoter [29], was a gift from M. Perry (University of Texas, Houston, TX, USA). 4Rtk-CAT, a CAT reporter construct driven by four reiterated MyoD binding sites (MEF1) upstream of a thymidine kinase (tk) promoter [30], was a gift from A. Lassar (Harvard University, Boston, MA, USA). For MyoD transactivation assays, pCSAMyoD plasmid was used [26]. Chicken MyoD (CMD1; [31]) and myogenin (a gift from B. Paterson, NIH, MD, USA) *Eco*RI inserts were excised with *Sac*II, yielding 500-nucleotide probes. A 250-nucleotide probe of chicken HGF (pCHA) was excised from a pKS (−) plasmid with *Eco*RI. A chicken c-met *Bam*HI–*Eco*RI insert (gift from T. Nohno, Kawasaky Medical School, Okayama, Japan) was excised from CMET1, giving a 250 bp probe [18]. Mouse HGF and c-met [32] were gifts from C. Ponzetto (University of Turine, Torino, Italy).

2.3. Animals and cell cultures

Chicken skeletal muscle satellite cells were cultured from the pectoral muscle of 4- to 5-day-old chicks as described by Halevy and Lerman [33]. In some experiments, cells were prepared from 12-day-old chicks. Cells were plated on 0.1% (w/v) gelatin-coated plates and maintained for the first 24 h in DMEM containing 1% (v/v) antibiotic–antimycotic solution, 10% (v/v) fetal calf serum (FCS), 10% (v/v) horse serum (HS) and 0.5% (v/v) chicken embryo extract (growth medium). Mouse C2 cells were supplemented with 20% (v/v) FCS. Cells were plated at 10^4 and 5×10^4 cells/cm^2 for C2 and satellite cells, respectively, and were induced to differentiate by changing the medium to DMEM containing 2% HS or 5% FCS, respectively (differentiation medium). Cells were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2.

2.4. Transient transfections

Proliferating primary chicken satellite cells were grown on 60 mm dishes and transfected with lipofectamine according to the manufacturer's directions. Plasmids were mixed in DMEM with 10 μl lipofectamine and added to the cells for 2 h, after which the medium was replaced with one containing 10% FCS. Differentiation medium was added 24 h after the addition of DNA to the cells and 24 h after that, cells were harvested for CAT assays as described [34]. Normalization of transfection efficiency was performed by correcting the CAT activity for the levels of constitutively expressed β-galactosidase (β-gal) plasmid [35]. Each transfection treatment was replicated three times with treatments run in triplicate.

2.5. MDCK-scattering activity

MDCK cells were seeded on 96-well plates (4×10^3 cells/well) and were incubated with various dilutions of factors in DMEM plus 5% FCS or 10% HS for 24 h. The scattering effect was monitored by light microscopy following Giemsa staining, as described by Stoker and Perryman [36].

2.6. Thymidine incorporation

DNA synthesis was assessed by [^{3}H]thymidine incorporation [33]. Cells were treated with the various factors for 20 h, then [^{3}H]thymidine was added (1 μCi/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 dissolved precipitates was counted in Quicksafe A scintillation fluid (Zinssen Analytic, UK) using a Tri-Carb 1600CA scintillation counter (Packard, Downers Grove, IL, USA).

2.7. Sarcomeric myosin heavy chain (MHC) expression

This assay was performed according to Johnson and Allen [37]. Briefly, cells were grown on 96-microtiter plates, fixed in methanol for 20 min, and incubated for 2 h with MF-20 (Developmental Studies Hybridoma Bank, University of Iowa; contributed by Dr. D. Fischman), a monoclonal antibody against sarcomeric myosin. Cells were then rinsed with PBS containing 0.05% (v/v) Tween-20, and incubated

with horseradish peroxidase sheep anti-mouse IgG (Amersham, UK). Color was developed in the presence of ABTS solution and hydrogen peroxide, and absorbance was recorded at 405 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Protein content was determined in parallel plates using the Pierce micro-protein assay according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

2.8. RNA preparation and hybridization

Total RNA was isolated as described previously [10,33]. The RNA was subjected to 1% agarose denaturing gel electrophoresis followed by blotting onto a nylon filter (GeneScreen Plus, New England Nuclear, Boston, MA, USA). cDNA probes were labeled using the random primer procedure [38] with a commercial kit (Boehringer Mannheim, Germany), and hybridization was performed overnight at 60°C in a solution containing 7% (w/v) SDS and 1% (v/v) BSA in 0.05 M phosphate buffer, pH 7.2. Filters were then washed and exposed to X-ray film (Agfa-Curix) at −70°C, using intensifying screens. Band intensity was quantified by densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. c-met and HGF are expressed in a reciprocal fashion during muscle cell differentiation

To better understand the role of HGF in skeletal muscle development and regeneration, we analyzed the expression of HGF mRNA and that of its receptor, c-met, and the biological activity of HGF in primary cultures of chicken satellite cells and the myogenic cell line C2. We used primary cultures because they are devoid of many of the problems associated with the use of cell lines, and may therefore represent a stage resembling the in vivo situation.

Proliferating myoblasts of both primary chicken satellite cell and mouse myogenic C2 cell cultures were grown in medium containing high serum levels, and then induced to differentiate by switching to differentiation medium for 48 h. Fusion percentage was between 80–85% in both cell cultures. Northern blot analysis revealed that c-met is expressed in proliferating chicken satellite cells (Fig. 1(A), day 1) and is slightly reduced on day 2, when cells begin to fuse (Fig. 1(A); [39]). Likewise, c-met mRNA was expressed in C2 myoblasts (Fig. 1(B)). These mRNA expression levels were reduced during cell differentiation in both cell lines, by approximately 2.5-fold and fivefold in satellite cells and C2 cells, respectively (Fig. 1(C)). The reduction in c-met levels in chicken satellite cells paralleled that of MyoD mRNA expression, previously shown to be reduced during the differentiation of these cells [10,39]. In contrast to c-met expression, HGF gene expression was not detected in proliferating myoblasts of either satellite cells or the C2 cell line (Fig. 1(A), days 1 and 2, Fig. 1(B)). Its induction was seen only when cells had differentiated and formed myotubes. However, HGF expression in myotubes was transient as it was not detected in the skeletal muscle tissue containing mainly mature myofibers (Fig. 1(D)).

To verify the expression and secretion of HGF protein by muscle cells, we used a biological assay for HGF activity in MDCK cells. These cells, which normally grow in islands, scatter in response to active HGF, thereby serving as model target cells for biological studies on HGF activity [36]. When conditioned medium (CM) collected from a culture of C2 myotubes (1:64 dilution) was added to MDCK cells, scattering activity was seen after 24 h of incubation (Fig. 2(B)), whereas control MDCK cells remained in islands (Fig. 2(A)). Some separation of MDCK cells was seen in the presence of C2 myoblast CM, albeit at a much lower dilution (1:2, data not shown). No effect on MDCK cells was observed when they were incubated in the presence of undiluted CM collected from cultures of either proliferating (data not shown) or differentiated chicken satellite cells (Fig. 2(C)). However, when CM collected from satellite cell myotubes was concentrated fivefold, some effect on MDCK cells was observed (Fig. 2(D)). Although cell separation was not complete, the islands were more spread out and there was a higher proportion of free cells, rendering them distinguishable from cells in the undiluted medium and control. To the best of our knowledge, HGF is the only factor known to have a scattering effect on MDCK cells. Thus, these results imply a correlation in both cell cultures between

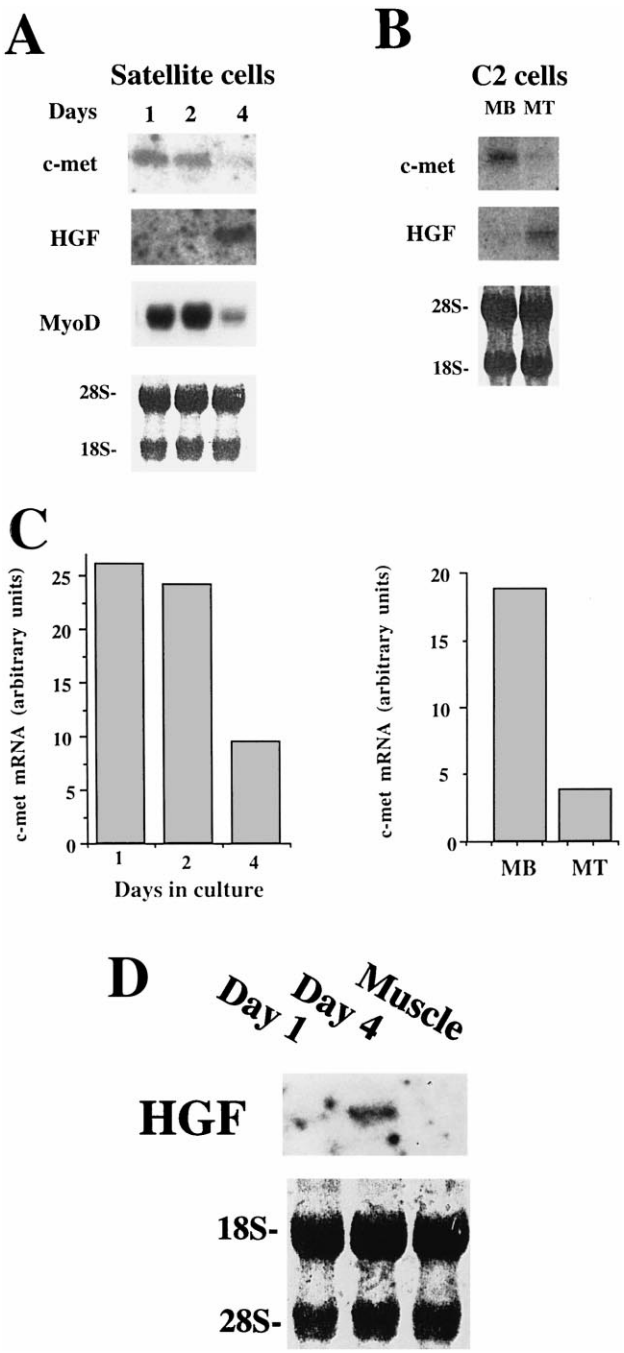

Fig. 1. Regulation of c-met and HGF mRNA expression during muscle cell differentiation. (A) Primary satellite cells were prepared and cultured in growth medium as described in Section 2. After 3 days in culture, the medium was switched to differentiation medium. RNA was prepared on the days indicated at the top of each lane. (B) Total RNA was prepared from either C2 myoblasts (MB) maintained in growth medium or myotubes (MT) maintained for 72 h in differentiation medium. Total RNA (20 μg/lane) was electrophoresed, blotted, and hybridized with the specific probes. Ribosomal RNAs were stained with methylene blue and 28S and 18S positions are indicated. (C) Quantification of changes in c-met mRNA in satellite cells (left panel) and C2 cells (right panel). Bands were quantified by densitometry and the level of c-met mRNA was expressed relative to that of ribosomal RNA. (D) Northern blot analysis of HGF mRNA expression in total RNA prepared from primary satellite cells on the days indicated at the top of each lane, and in muscle tissue.

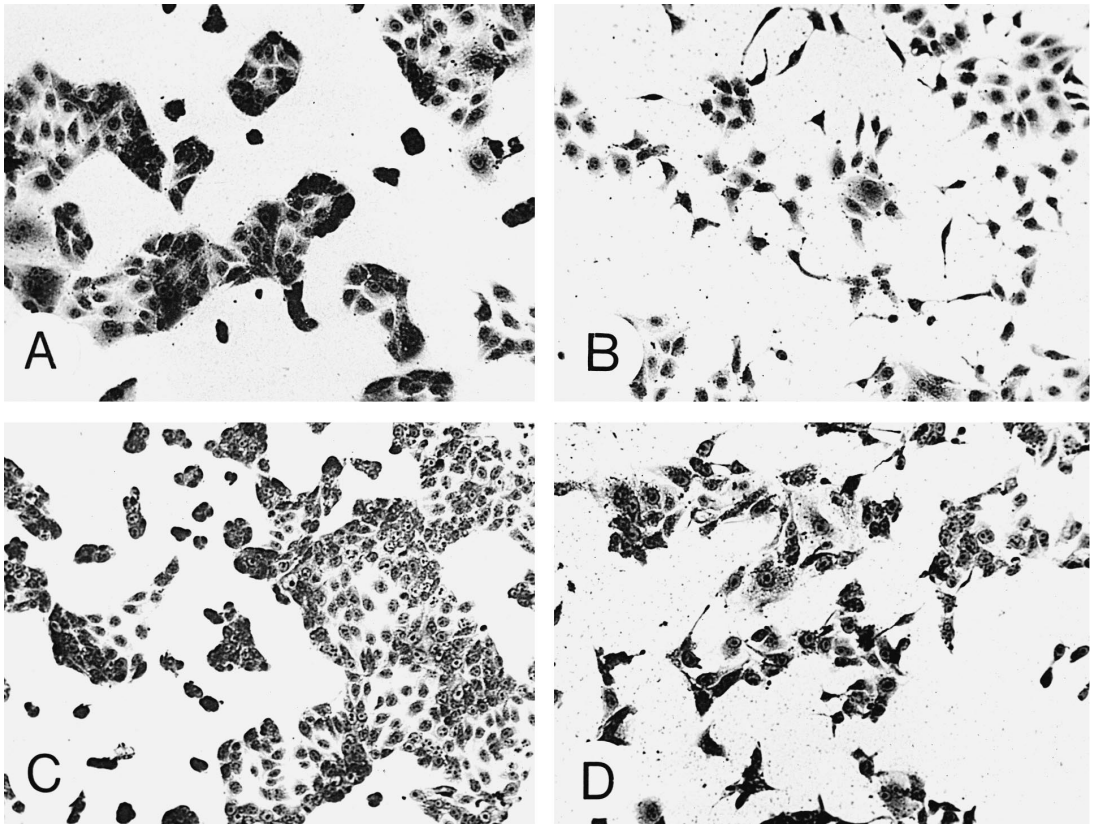

Fig. 2. Distribution of MDCK cells in assay-plate wells without (A) or with CM derived from C2 myotubes (1:64 dilution; B), and non-concentrated (C) or fivefold concentrated (D) CM derived from chicken satellite cells. Plates show wells with MDCK cells after 24 h in the presence of the various media.

HGF gene expression and its secretion as an active protein into the medium. It should be noted that in this case the MDCK assay was a qualitative rather than a quantitative one, therefore, the low effect of CM derived from chicken satellite cell myotubes on MDCK cells may be due to that. Another explanation could be due to relatively low homology between rat and human HGFs and chick HGF [74% at the deduced protein level; 19]. However, the MDCK cell response was markedly enhanced in CM obtained from chicken satellite cell cultures in which ectopic HGF was overexpressed (see Fig. 5(A)).

3.2. Exogenous HGF affects satellite cell DNA synthesis and increases c-met gene expression

The effect of recombinant human HGF on muscle cell proliferation was evaluated in primary chicken satellite cells. After 1 day in culture in growth medium, the satellite cells were maintained for 24 h in 0.5% FCS-containing medium. This medium was then switched to one containing recombinant HGF at various concentrations for an additional 24 h. As indicated in Fig. 3(A), HGF increased satellite cell DNA synthesis in a dose-dependent manner to a peak in cells incubated with 20 ng/ml HGF. Although higher concentrations of HGF slightly reduced DNA synthesis, it remained substantially higher than in the control, untreated cells.

HGF's ability to activate quiescent chicken satellite cells was addressed in cells prepared from 12-day-old chicks: these were considered quiescent cells, since in chicken, most of these cells are active only during the first week of life [10,40]. Satellite cells were incubated from time of plating with medium containing 10% HS, with or without human HGF (20 ng/ml), and mitogenic concentrations established in our laboratory of human bFGF (1 ng/ml; [39]) or

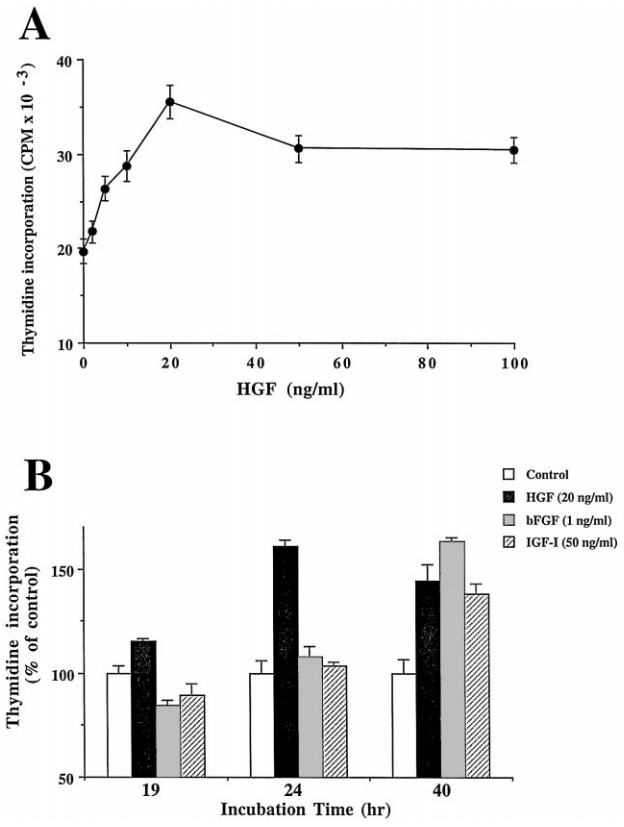

Fig. 3. (A) Dose-dependent effect of HGF on DNA synthesis. Cells were incubated with or without HGF at various concentrations in medium containing 0.5% FCS for 17 h and [^{3}H]thymidine was added for an additional 4 h. Results are means ± SEM of six replicates. (B) Effect of HGF and various growth factors on satellite cell DNA synthesis. Cells were prepared from 11-day-old chicks and seeded in DMEM containing 10% HS in the absence or presence of human HGF (20 ng/ml), human bFGF (1 ng/ml) or chicken IGF-I (50 ng/ml) for the indicated time points. [^{3}H]Thymidine was added for the last 2 h of incubation for each time point. Results are means ± SEM of six replicates.

chicken IGF-I (50 ng/ml; Hodik et al., unpublished results). Entry of cells into the S phase of the cell cycle was monitored by thymidine incorporation into DNA and is presented as percent of control, untreated cells (Fig. 3(B)). After 19 h of incubation, a slight increase in thymidine incorporation was seen in cells exposed to HGF relative to control cells, whereas in the case of bFGF and IFG-I, the levels of DNA synthesis were even lower than in the control. After 24 h of incubation, HGF's effect on thymidine incorporation was more pronounced, with up to 1.6-fold higher incorporation than in control cells, whereas the other factors did not have any effect on DNA synthesis. Only after 40 h in culture was increased, DNA synthesis seen in the presence of all growth factors, this increase being highest in cells incubated with bFGF. Taken together, these results imply that the effect of HGF on DNA synthesis and cell proliferation is due to earlier entry into the cell cycle.

3.3. The effect of exogenous HGF on sarcomeric MHC expression in satellite cells

Expression of a muscle-specific marker, sarcomeric MHC, was evaluated in chicken satellite cells in the presence of recombinant human HGF in dose-response experiments. The experiments were performed in the presence of 5% FCS, this serum level being low enough to induce differentiation [10]. Primary chicken satellite cells were grown for 1 day in growth medium, then switched to medium containing 5% FCS with or without recombinant human HGF (20 ng/ml). MHC protein levels were reduced in a dose-dependent manner when exposed to increasing levels of HGF, up to 50 ng/ml (Fig. 4), suggesting that exposure of satellite cells to HGF inhibits their differentiation, probably by affecting muscle-specific gene expression.

3.4. Ectopic HGF inhibits transactivation of muscle-specific factors in primary satellite cells

To investigate the molecular pathways involved in HGF-inhibited myogenesis, we chose to examine the

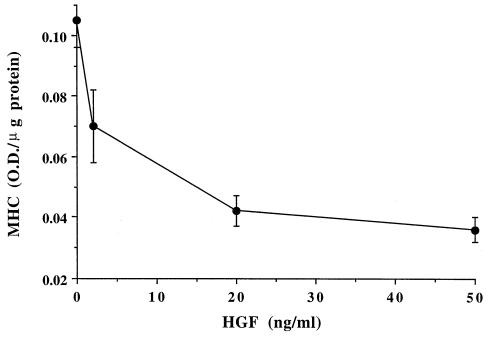

Fig. 4. HGF inhibition of chicken muscle satellite cell differentiation. Expression of sarcomeric MHC in satellite cells incubated for 48 h in the presence of various concentrations of recombinant human HGF in differentiation medium. Results represent means ± SEM of 6 replicates.

effects of the homologous chicken HGF on primary cultures of proliferating chicken satellite cells. Cells were transiently co-transfected with expression vectors encoding chicken HGF under the control of the cytomegalovirus promoter (pCSAcHGF; 1.5 μg) or vehicle alone (pCSA; 1.5 μg). Two days post-trans-

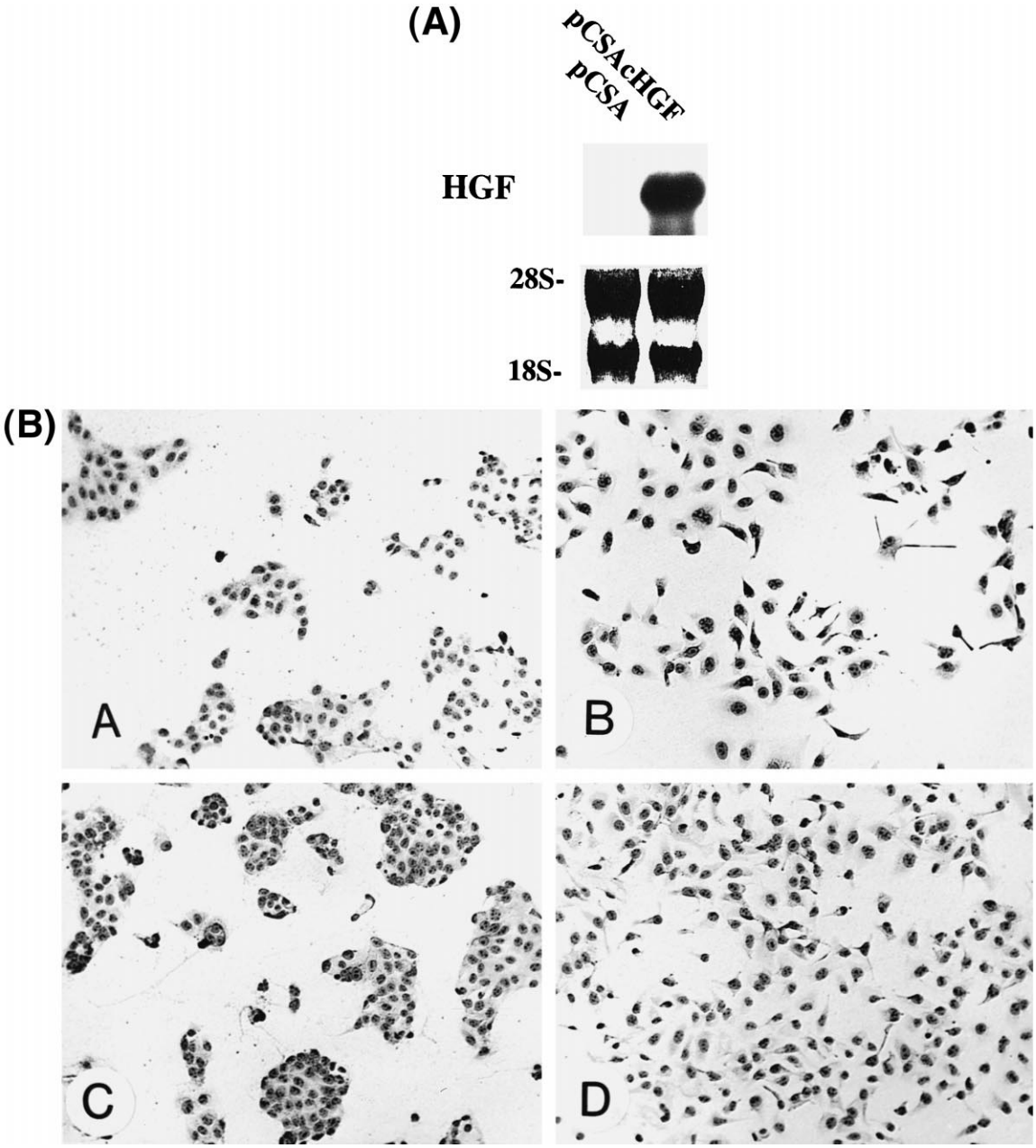

Fig. 5. Effect of ectopic HGF on HGF expression in primary satellite cells. (A) Expression of HGF mRNA in satellite cells transfected with pCSA or pCSAcHGF (1.5 μg). Northern blot analysis was performed as described in Section 2. Ribosomal RNAs were stained with methylene blue and 28S and 18S positions are indicated. (B) Primary chicken satellite cell myoblasts were transiently transfected with pCSA or pCSAcHGF (1.5 μg). After 24 h in differentiation medium, CM was collected and added to MDCK cells as described in Section 2. Plates show MDCK cells exposed to 10% FCS (A), 20 ng/ml human HGF (B), CM of vector-transfected cells (C) and pCSAcHGF-transfected cells (D).

fection, a marked induction in the expression levels of HGF mRNA was observed in cells transfected with pCSAcHGF vs. vehicle-transfected cells (Fig. 5(A)). This result was confirmed by analyzing the expression and secretion of active chicken HGF by transfected cells via MDCK cell-scattering (Fig. 5(B)). CM collected from either control, untreated or vehicle-transfected satellite cells exhibited no scattering effect (Fig. 5(B), plates A and C). However, CM derived from pCSAcHGF-transfected cells (1:16 dilution) had a scattering effect on MDCK cells similar to that achieved with the addition of 20 ng/ml recombinant HGF (Fig. 5(B), plates B and D), indicating several-fold higher levels of ectopic HGF. Together, these results indicate a substantial increase in HGF mRNA and protein levels in satellite cells which can be attributed mainly to ectopic HGF, as endogenous HGF levels secreted into the medium were very low in these cells (see Fig. 2).

The effect of ectopic HGF on the activation of various muscle-specific promoters was examined in cells that were co-transfected with pCSAcHGF and a CAT reporter driven by the muscle creatine kinase promoter (MCK-CAT), together with 0.1 μg β-gal which was used as a control for transfection efficiency. When transfected into satellite cells, pCSAcHGF reduced the activation of MCK-CAT in a dose-dependent manner (Fig. 6(A), lanes 1–4) and a maximal reduction to approximately 35% transactivation was observed with 1.5 μg DNA of ectopic chicken HGF (Fig. 6(A), lane 4). This reduction was not due to pCSAcHGF toxicity because its co-transfection with a non-muscle-specific promoter (pCSA-CAT) did not cause any reduction in CAT activity relative to that in vehicle-transfected cells (Fig. 6(A), lanes 5 and 6).

We then examined the effect of ectopic chicken HGF on the activation of muscle-specific regulatory sites associated with chicken MRF4. This 336 bp 5′-flanking sequence has been shown to be sufficient to generate a muscle-specific transcription response [28]. Co-transfection of either −336MRF4-CAT or −3300MCK-CAT and pCSAcHGF into chicken primary cultures revealed a twofold reduction in CAT activation, as compared to that in the pCSA-transfected cells (Fig. 6(B), lanes 1–4). A similar reduction in CAT transactivation was obtained when HGF was co-transfected with reiterated MEF2 binding sites

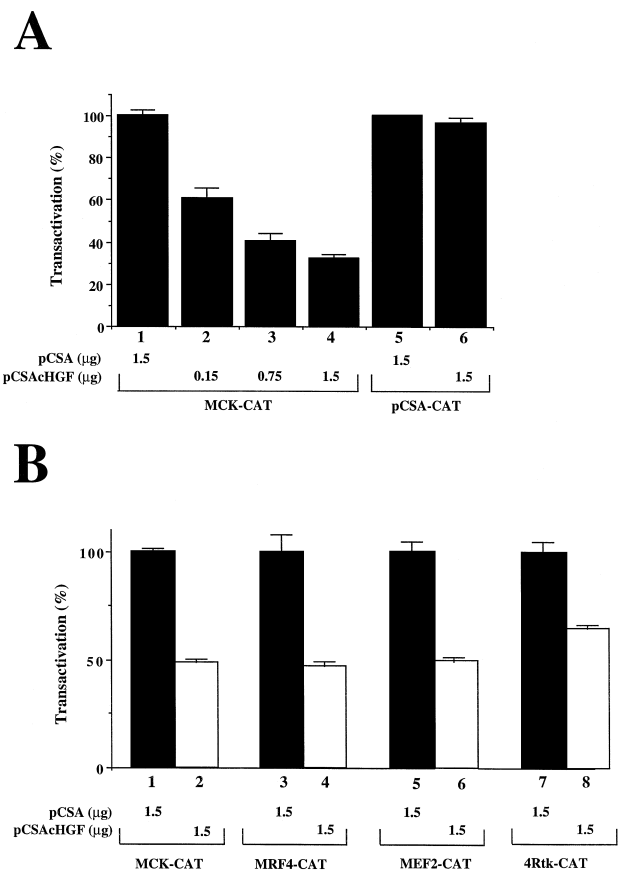

Fig. 6. (A) Transfection of satellite cells with various concentrations of pCSAcHGF, 0.1 μg β-gal and 0.5 μg MCK-CAT (lanes 1–4), or 0.5 μg pCSA-CAT (lanes 5 and 6). Cultures were harvested after 48 h post-transfection, and CAT activity was determined and normalized to β-gal activity. (B) Inhibition of muscle-specific gene activation by ectopic chicken HGF. Proliferating muscle satellite cells were transiently co-transfected with equal amounts of pCSAcHGF and β-gal (0.1 μg), and either 0.5 μg MCK-CAT (lanes 1 and 2), MRF4-CAT (lanes 3 and 4), MEF2-CAT (lanes 5 and 6) or 4Rtk-CAT (lanes 7 and 8). Bars represent CAT activity relative to that of vehicle-transfected cells. Each value represents the mean and SEM of triplicate results. Experiments were replicated twice with similar results.

driving Xenopus MyoD1 promoter (Fig. 6(B), lanes 5 and 6). Transcriptional activation of reiterated MyoD binding site (MCK enhancer MEF1 site) upstream of the thymidine kinase (tk) promoter (4Rtk-CAT) was also reduced to approximately 65% transactivation by ectopic HGF (Fig. 6(B), lanes 7 and 8). Taken together, these results imply a specific role for HGF in the regulation of the myogenic differentiation program by its effect on MDF/E-protein complexes.

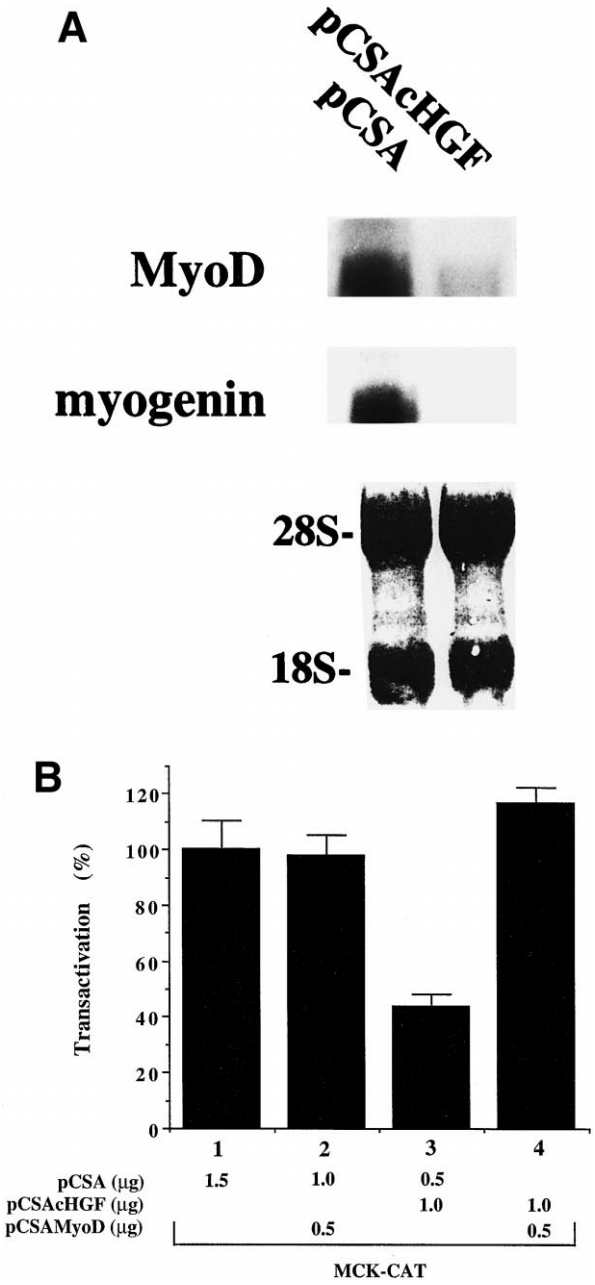

Fig. 7. (A) Ectopic HGF suppresses muscle-regulatory gene expression. Total RNA extracted from satellite cells transiently transfected with vehicle alone or ectopic chicken HGF was analyzed by northern blotting with MyoD and myogenin probes. RNA integrity is shown by methylene-blue staining of ribosomal RNA. (B) Reversal of HGF-mediated inhibition of MCK transactivation by ectopic MyoD. Satellite cells were transiently transfected with 0.5 μg MCK-CAT plus vehicle alone (lane 1), pCSAMyoD (lane 2), pCSAcHGF (lane 3), or pCSAcHGF and pCSAMyoD (lane 4). CAT activity is displayed as percent activity relative to that observed with empty plasmid vehicle. Bars represent the mean and SEM of triplicate results. Experiments were replicated twice with similar results.

3.5. Ectopic HGF represses muscle-regulatory and muscle-specific factors in satellite cells

We next investigated the relationship between the action of HGF on the transactivation of muscle-regulatory factors and the expression of myogenic factors. In the presence of ectopic chicken HGF, the protein levels of MHC in satellite cells were approximately 2.5-fold lower than that in cells transfected with vehicle alone (0.11 vs. 0.26 O.D./μg protein, respectively). Furthermore, in a northern blot analysis of total RNA harvested from the transiently transfected primary satellite cells, MyoD and myogenin mRNA expression levels were substantially reduced in pCSAcHGF-transfected cells relative to vehicle-transfected cells (Fig. 7(A)).

To ascertain whether HGF directly affects MyoD activity, we assayed the ability of ectopic MyoD to reverse the HGF-mediated inhibitory effect on MCK transactivation in satellite cells (Fig. 7(B)). When compared to vehicle-encoding plasmid transfection, ectopic MyoD did not effect any major change on the transactivation of MCK promoter (Fig. 7(B), lanes 1 and 2). As expected, transfection of pCSAcHGF in the absence of ectopic MyoD caused a reduction of approximately 55% in MCK-CAT activity (Fig. 7(B), lane 3). However, when pCSAcHGF was co-transfected with pCSAMyoD, activation of the MCK-CAT reporter was boosted to higher levels than that achieved in the control cells (Fig. 7(B), lanes 4 vs. 1, respectively). The ability of ectopic MyoD to reverse the inhibitory effect of HGF on MCK-CAT reporter activity suggests that HGF's effect is at least partially MyoD-mediated.

4. Discussion

In regenerating or growing adult skeletal muscle tissue, there is a need for as many new precursor cells, i.e. satellite cells, as possible. This need can be manifested in a variety of ways, under the influence of local growth factors, in an autocrine/paracrine fashion. One way is to activate quiescent satellite cells, driving them into the cell cycle. Previous studies have demonstrated that various mitogens do not activate quiescent satellite cells in culture [41], or on isolated muscle fibers [6]. Our results demonstrate

that HGF, and not other mitogens such as bFGF and IFG-I, is the first to bring primary satellite cells into the S phase of the cell cycle. In addition to its function as an activator, added HGF was able to dose-dependently increase the proliferation of satellite cell myoblasts in a primary culture. This mitogenic effect is probably common in regenerating tissues as it has been seen in many cell types, particularly those participating in tissue regeneration such as hepatocytes and kidney tubular epithelial cells (reviewed in [15]). Taken together, our and other's results suggest that HGF is an activator for quiescent satellite cells and a mitogen during muscle regeneration.

Another way of achieving a large number of precursor myogenic cells is to delay the satellite cell differentiation program, thereby allowing more cells to proliferate and eventually add more fibers. The addition of exogenous HGF to satellite cells dose-dependently inhibited the expression of MHC, which is expressed late in muscle cell differentiation. A substantial effect on the expression of muscle-regulatory genes was seen in the presence of ectopic chicken HGF, which was secreted at high levels by transfected satellite cells (see Fig. 5(B)). Furthermore, ectopic chicken HGF reduced the transactivation of the muscle-specific MCK promoter in a dose-dependent manner, as well as the transactivation of chicken MRF4 promoter (both containing an E-box and MEF2 sites; [27,28]), and artificial reporters containing four reiterated MEF2 binding sites or E boxes. Our assumption is that HGF inhibits muscle cell differentiation by inhibiting the activity of MDF/E protein heterodimers that are able to bind E-box or MEF2 sites. In support of this we demonstrated that transfection of MyoD-encoding plasmid could reverse the inhibitory effect of HGF on MCK promoter transactivation.

HGF production in regenerating tissues such as liver and lung following injury has led to the suggestion that it is physiologically important for the control of organ regeneration [10,42]. Our findings, together with those of others, further support HGF's important role in skeletal muscle regeneration. Nevertheless, HGF's mode of action in this process has not been completely elucidated. In this study, HGF mRNA expression was barely detectable in C2 and chicken satellite cell myoblasts and was elevated only when cells fused and formed myotubes. HGF secretion could not be attributed to the myofibers as its gene expression in skeletal muscle tissue was abolished. These data disagree with a recent study which shows that HGF is down-regulated during muscle cell differentiation [24]. However, our data support earlier studies in which in-situ hybridization of HGF in regenerating muscle in rat revealed its expression in early myotubes, not in mature fibers [16]. In that study, there was evidence for HGF mRNA expression in myoblasts also, and it is possible that this discrepancy with our results may be due to species differences. In addition, transcripts of c-met and HGF were never found in the same cells in muscle during mouse development [17]. In contrast to HGF, mRNA expression levels of its receptor, c-met, were reduced during cell differentiation of both C2 and satellite cells and were very low in myotubes. Our notion, is that upon muscle injury, HGF, initially secreted by surrounding cell types (e.g. fibroblasts), activates quiescent satellite cells recently shown to express c-met [25]. These enter the cell cycle, proliferate and undergo terminal differentiation, leading to the activation of additional satellite cells by HGF secreted by the newly formed myotubes. This HGF may also act as a potent mitogen for myoblasts since they exhibit c-met expression. In addition, it delays cell differentiation, thus allowing more cells to proliferate. For instance, DNA synthesis in ectopic cHGF-transfected satellite cells was 1.6-fold higher than that in vehicle-transfected cells, even after 2 days in low serum-containing medium (data not shown). After withdrawal from the cell cycle and cell differentiation, c-met expression is suppressed in satellite cells, and hence they no longer respond to HGF. The HGF secreted from muscle cells may also affect other cell types, such as endothelial cells.

In view of these findings, it is possible that during muscle regeneration in vivo, myotube-secreted HGF acts on proliferating myoblasts and other cell types mainly via a paracrine mechanism. A paracrine effect of HGF has been suggested in early development: when secreted from mesenchymal cells, it regulates the development of several epithelial and myogenic precursor cells during organogenesis [17,43]. HGF's paracrine mode of action has also been suggested in other cell types, in the proliferation of human glioblastoma cells [44], and in the prostate gland,

where HGF secreted by stromal cells affects epithelial cell proliferation [45]. Nevertheless, the possibility that HGF can exert its effects on muscle cells via an autocrine/paracrine pathway cannot be ruled out, since exogenous and ectopic HGF induces c-met mRNA levels, which may enable these cells to respond to HGF (Gal-Levi and Halevy, unpublished results). In addition, an autocrine loop has been suggested recently for HGF action on C2 cells [24].

In postnatal muscle, satellite cells are known to have extensive powers of migration, and during regeneration after muscle damage, satellite cells detach from the surface of fibers and migrate within the muscle [46]. Previous studies have indicated a role for HGF and its receptor in myoblast migration to the limb bud [21], and an effect on satellite migration in culture [47]. Therefore, it may well be that HGF affects satellite cell migration during muscle growth and regeneration.

In conclusion, our findings suggest that HGF and its receptor, c-met, play an important role in both promoting muscle satellite cell proliferation and inhibiting their differentiation via inhibition of muscle-regulatory-factor activity. The reciprocal expression of HGF and c-met genes during satellite cell differentiation suggests a paracrine mode of action of HGF in these cells.

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