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Research Article

Pleiotropic effects of sonic hedgehog on muscle satellite cells

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Abstract. Muscle satellite cells are believed to form a stable, self-renewing pool of stem cells in adult muscle where they function in tissue growth and repair. A regulatory disruption of growth and differentiation of these cells is assumed to result in tumor formation. Here we provide for the first time evidence that sonic hedgehog (Shh) regulates the cell fate of adult muscle satellite cells in mammals. Shh promotes cell division of satellite cells (and of the related model C2C12 cells) and prevents their differentiation into multinucleated myotubes. In addition, Shh inhibits caspase-3 activation and apoptosis induced by serum deprivation. These effects of Shh are reversed by simultaneous administration of cyclopamine, a specific inhibitor of the Shh pathway. Taken together, Shh acts as a proliferation and survival factor of satellite cells in the adult muscle. Our results support the hypothesis of the rhabdomyosarcoma origin from satellite cells and suggest a role for Shh in this process.

Key words. Sonic hedgehog; muscle satellite cell; proliferation; differentiation; apoptosis; rhabdomyosarcoma.

During development, the morphogen sonic hedgehog (Shh) most commonly affects cell fate, but it can also affect cell growth and cell survival [1]. Postnatally, several distinct cancers result from mutations of the Shh receptor *Patched1* (*Ptch1*), which mimic activated Shh signal transduction. Aberrant Shh signaling is thought to affect pools of cancer-specific progenitor cells. This has been suggested for prostate carcinoma, for small-cell lung cancer, for tumors of the gastro intestinal (GI) tract, for medulloblastoma (MB), and for basal cell carcinoma (BCC) [2–6].

Abnormal Shh signaling may also lead to embryonal rhabdomyosarcoma (RMS), which is frequently observed in mice that lack one functional allele for *Ptch1* (*Ptch1*$^{neo67/+}$). Due to its function as an antagonist of Shh signaling, *Ptch1* mutations lead to a pathological activation of the pathway, which is evidenced by overexpression of its targets *Gli1* and *Ptch1* itself [7, 8]. The cell of origin for embryonal RMS is unknown, but a credible candidate is the satellite cell of the skeletal muscle [9, 10].

Satellite cells are adult skeletal muscle precursor cells that reside in indentations between the plasma membrane and the basal lamina of the muscle fiber [11]. These cells are believed to form a stable, self-renewing pool of stem cells. In the adult muscle, the satellite cells are normally quiescent. Following damage of the muscle, they are activated and contribute to the regeneration of the organ. This process consists of a short proliferation phase, followed by cell cycle withdrawal and differentiation into myoblasts, and by final fusion of the latter with neighboring myotubuli [11]. During proliferation, satellite cells express the myogenic factors MyoD and Myf5, whereas myogenin and Mrf4 function in the following differentiation into myoblasts. External growth factors including hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGFs) and transforming growth factor-β (TGF-β) play a role in the activation

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of satellite cells [12]. Nevertheless, the molecular mechanisms that control proliferation and differentiation of satellite cells are far from being completely understood and more factors or signaling pathways are assumed to be involved [13].
We hypothesized that mammalian satellite cells undergo proliferation activation in response to Shh. Since the response of these cells to Shh has not been investigated, we exposed murine satellite cells to this substance and measured their proliferation, differentiation, and apoptosis. In parallel, we performed these experiments on C2C12 cells, which are myoblasts capable of differentiation into contracting myotubes under low serum concentrations or when grown to confluence [14].

Materials and methods

Reagents

N-active sonic hedgehog protein (N-Shh) was kindly provided by Curis (Cambridge, Mass.) and was used at a concentration of 1 μg/ml unless otherwise indicated. Due to the limited amount of N-Shh, in several pilot experiments we also used recombinant amino-terminal mShh peptide from R&D Systems (Wiesbaden, Germany). Dulbecco's modified Eagle's medium (DMEM) was from PAN Biotech (Nuernberg, Germany), fetal calf serum (FCS), horse serum (HS) and penicillin/streptomycin (Pen/Strep) were purchased from Invitrogen (Karlsruhe, Germany). The cyclopamine derivate 3-keto-N-aminoethyl aminocaproyl dihydrocinnamoyl cyclopamine (KAAD-cyclopamine) was obtained from Toronto Research Chemicals (Toronto, Canada) and was used at a final concentration of 1 μM.

Animals

Satellite cells, RMS and non-cancerous skeletal muscle (SM) were obtained from wild-type or heterozygous *Ptch1*$^{neo67/+}$ mice maintained on a CD-1 background [7]. All mice were treated and housed in accordance with the German animal protection laws and monitored regularly by the campus veterinarian.

Cell culture

Primary muscle satellite cells were isolated from hind limb muscle and diaphragm of 6- to 8-week-old wild-type and heterozygous *Ptch1*$^{neo67/+}$ mice according to protocols described by Allen et al. [15] and Yablonka-Reuveni and Nameroff [16], with modifications. Briefly, after dissection and mincing, muscle tissue was digested using 1.25 mg/ml Pronase E (Merck Eurolab, Darmstadt, Germany) for 1 h at 37 °C. During enzyme digestion, the cell suspension was triturated every 15 min using a sterile 5-ml pipette in order to facilitate satellite cell detachment from myofibers. The cells were washed by centrifugation for 3 min at 2000 g, resuspended in prewarmed DMEM/10% FCS/1% PenStrep and filtered through a 100-μm cell filter. Next, satellite cells were separated from fibroblasts on a Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) by centrifugation for 20 min at 2500 g at 25 °C. The satellite cell fraction was collected on the 40%/70% surface and washed with PBS. The cells were resuspended in prewarmed differentiation medium (see below) and preplated onto an uncoated plate to remove the contaminating highly adhesive fibroblasts. One hour later, the supernatant enriched with satellite cells was transferred onto collagen-coated plates with proliferation medium (see below).
For most experiments, muscle satellite cells were grown for 5 days on collagen-coated plates in DMEM containing 30% FCS/10% HS/1% Pen/Strep (growth medium for satellite cells), which was then switched to DMEM supplemented with 10% HS and 1% Pen/Strep only (differentiation medium for satellite cells). Medium was changed every day. The identity of satellite cells was confirmed by staining with a polyclonal rabbit c-met antibody (SP260; Santa Cruz Biotechnology, Heidelberg, Germany; see below).
Mouse myoblast C2C12 and fibroblast NIH-3T3 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) and maintained in DMEM/10% FCS/1% Pen/Strep (growth medium for C2C12 cells). In addition, experiments with C2C12 were performed in DMEM/2% HS/1% Pen/Strep (differentiation medium for C2C12).

Proliferation assay

To assess the growth rate of primary satellite cells, a commercially available cell proliferation ELISA (Roche Diagnostics, Mannheim, Germany) was used. Cells were cultivated in the respective differentiation medium on 96-well plates, then treated for 24, 48, 72, 96, or 120 h with 0.5–4 μg/ml Shh (stock: 250 μg/ml in 0.1% BSA/PBS) in the presence and absence of 1 μM cyclopamine (stock: 1 mM in methanol) or with 5 ng/ml HGF (stock: 1 μg/ml in 0.1% BSA/PBS). All control cells were treated with an equal amount of the respective vehicle. 5-Bromo-2′-deoxyuridine (BrdU) was added to the culture medium to a final concentration of 10 μM for the last 24 h of the experiment. After removing the BrdU-containing medium, the cells were fixed and stained with a peroxidase-coupled anti-BrdU antibody according to the manufacturer's protocol. After 90 min, the samples were washed, substrate solution was added and luminescence was measured.
To exclude falsifications due to toxic effects of Shh and/or cyclopamine, the protein concentration of the plated cells was measured in a control experiment using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany).

Table 1: Gene-specific primers used for semi-quantitative RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size
Desmin	tcc ggt acc aag ggc tcc tcg agt t	tca cgg atc tcc tct tca tgc act	625 bp
Gapd	atc ttc ttg tgc agt gcc a	atg gca tgg act gtg gtc at	576 bp
Gli1	ttt-gcc acc aag cca act tta tg	agt ctg tct cat aca cag act ca	599 bp
M-cadherin	gtg cag atc aag tct gac aaa cag	atc atc tat gga gat gat ggc cga	560 bp
Pax7	cca aga ttc tgt gcc gat atc agg a	agc tgg tta gct cct gcc tgc tta	585 bp
Ptch1	ttc tgc tgc ctg tcc tct tat c	cag aat gcc ctt cag tag aa	492 bp
Shh	atg agg aaa aca cgg gag cag a	ggt tga tga gaa tgg tgc cgt gc	764 bp

To compare BrdU incorporation between wild-type and *Ptch1*$^{neo67/+}$ satellite cells, experiments were repeated three times in triplicate. In addition to counting the cell number prior to transfer into differentiation medium, protein concentration was measured in a control experiment.

Immunofluorescence and TUNEL assay

To examine myogenin expression, primary satellite cells and C2C12 cells were grown in the respective differentiation medium with and without Shh and/or cyclopamine on culture slides for 120 h.

For the TUNEL assay and for measurement of active caspase 3, C2C12 cells were grown for 24 h in proliferation medium and then switched to differentiation or serum-free medium for the next 24, 48, 72, and 120 h in the presence or absence of Shh and cyclopamine.

For antibody staining, cells were fixed in fresh 4% paraformaldehyde for 20 min at room temperature, permeabilized in 0.1% Triton-X100/0.1% sodium citrate, blocked in 2% BSA and incubated overnight at 4 °C with polyclonal rabbit anti-human/mouse Caspase 3 active antibody (0.3 μg/ml; R&D Systems, Wiesbaden, Germany), polyclonal rabbit c-met antibody SP260 (1:200; Santa Cruz Biotechnology, Heidelberg, Germany), or monoclonal mouse anti-myogenin antibody F5D (5 μg/ml; BD Biosciences Pharmingen) in blocking solution. After incubation with TRITC-conjugated anti-rabbit (1:400) or FITC-conjugated anti-mouse (1:100) secondary antibodies, the samples were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, Calif.). Fluorescent signals were captured using a BX60 microscope (Olympus Optical Co., Tokyo, Japan) and the AnalySIS software (Soft Imaging System, Muenster, Germany). All photographs were made at ×200 magnification. Merged images were generated using Adobe Photoshop 6.0 software.

For TUNEL assay, cells were fixed, permeabilized, and washed as described above. Cells were incubated for 60 min at 37 °C with TUNEL label and enzyme as recommended by the manufacturer (Roche Diagnostics).

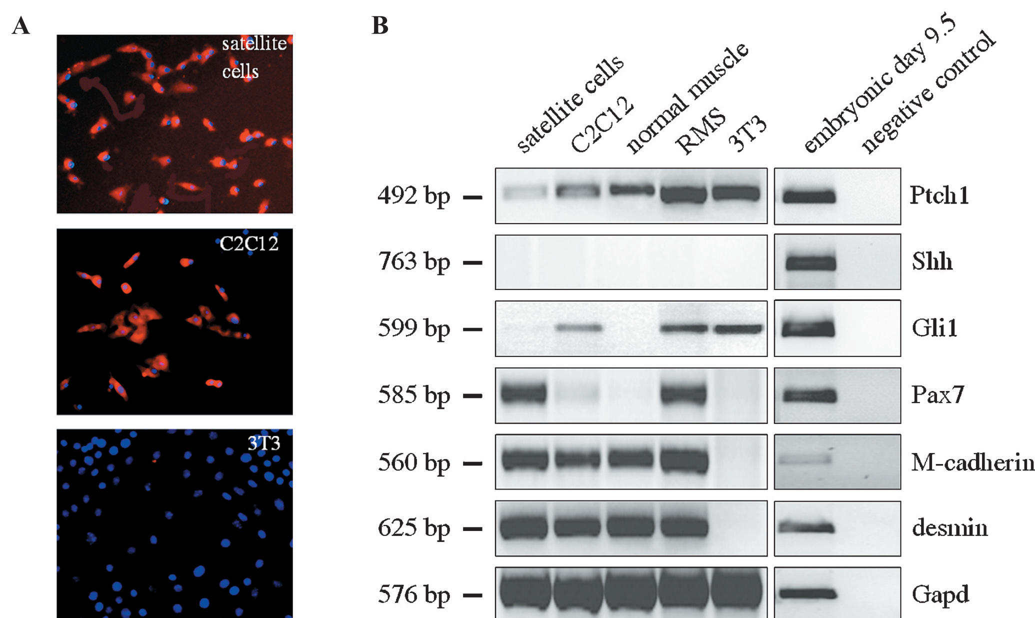

Figure 1. Expression of c-met protein (*A*) and of satellite cell-specific markers and components of the Shh signalling cascade (*B*) in satellite, C2C12, and 3T3 cells, normal muscle and an RMS of a *Ptch1*$^{neo67/+}$ mouse. cDNA derived from 9.5-day-old mouse embryos was used as positive control.

After washing with PBS, slides were mounted as described above. All experiments were assayed in triplicate.

Western blot analysis

For Western blot analysis, C2C12 cells were grown with and without serum in the presence or absence of Shh for 72 h. The cells were washed with PBS, harvested in an SDS lysis buffer (1% SDS, 10 mM Tris/HCl pH 7.4, 1 mM sodium vanadate) and boiled for 10 min. The protein amount was measured with a commercial assay (Bio-Rad). Equal amounts of protein were separated on an SDS-4–20% polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham Pharmacia). After blocking for 1 h at room temperature with 5% dry milk/PBS/0.1% Tween 20 (PBS-MT), immunodetection was performed by incubating the membranes with a mouse anti-caspase 3 antibody (Cell Signaling Technology, Beverly, Mass.) at a dilution of 1:1000 in PBS/0.1% Tween 20/2% BSA overnight at 4 °C. A mouse anti-β-actin antibody (1:10,000 in PBS/0.1% Tween 20/2% BSA; Sigma, Deisenhofen, Germany) was used to confirm equal loading of protein and blotting in each line. After washing in PBS/0,1% Tween 20, peroxidase-conjugated second antibody (goat anti-rabbit HRP from Santa Cruz Biotechnology, Santa Cruz, Calif.) was added (1:5000 in PBS-MT) for 1 h at room temperature. The membranes were washed three times in PBS/0.1% Tween 20, and blots were developed with an ECL chemiluminescence detection system (Amersham Pharmacia).

RT-PCR

Total RNA of satellite cells, C2C12 cells, 3T3 cells and of RMS and normal SM of the hind limbs of heterozygous *Ptch1*$^{neo67/+}$ mice was isolated using Trizol Reagent (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol. Two micrograms total RNA was reverse-transcribed using random hexamers (Roche Diagnostics) and SuperScript II (Life Technologies) and the cDNAs were amplified with the gene-specific primers listed in table 1 that were designed to cross introns to distinguish genomic DNA amplification. Amplification of *Gapd* as an endogenous control was performed to standardize the amount of sample RNA.

Results

The isolated cells were confirmed as satellite cells by immunostaining using a c-met antibody (fig. 1a). Similarly to RMS isolated from *Ptch1*$^{neo67/+}$ mice and to the myoblast cell line C2C12, they expressed the satellite cell-specific markers *pax7*, *M-cadherin,* and *desmin*. In contrast, these markers were not detectable in 3T3 fibroblasts (fig. 1b). Low levels of *Ptch1* and *Gli1* transcripts were expressed both in satellite cells and C2C12 cells, whereas *Shh* transcripts were not (fig. 1b). Exposure of satellite cells and C2C12 cells to 1 µg/ml of Shh for 72 h increased the expression of *Ptch1* and/or *Gli1* transcripts (fig. 2). These effects were effectively blocked by cyclopamine, which is a highly specific inhibitor of the pathway [17] (fig. 2).

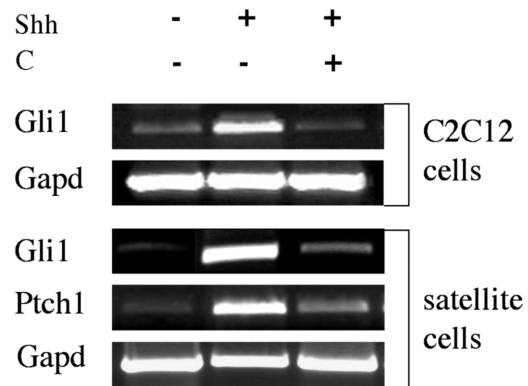

Figure 2. Induction of *Gli1* and/or *Ptch1* expression by Shh in C2C12 and primary satellite cells. The specificity of the induction is demonstrated by concomitant treatment of the cells with cyclopamine (C), a specific inhibitor of Shh signalling. *Gapd* was used as an internal control.

Next, we investigated the effect of Shh on cell proliferation. Since the effects of Shh vary with concentration [18], we first measured BrdU incorporation at four concentrations (0.5, 1, 2, and 4 µg/ml) of Shh (from R&D) in C2C12 cells (fig. 3a). The effect of Shh was both concentration and time dependent. After 72 h of incubation with Shh, an increase in BrdU incorporation was observed already at a concentration of 0.5 µg/ml and it was highest at 4 µg/ml. At the latter concentration, the BrdU incorporation peaked at 72 h (fig. 3b).

Primary satellite cells from wild-type mice were then exposed to 1 µg/ml of Shh for 72 h. As shown in figure 3c, this led to a three- to fourfold increase in BrdU incorporation in comparison to untreated cells. The extent of the BrdU effect was thus similar to that of 5 ng/ml HGF (four- to fivefold, data not shown), which is a potent mitogen in satellite cells [19]. The effect of Shh was halved in the presence of 1 µM cyclopamine, indicating that the effect of Shh was mediated through the Hedgehog signaling cascade (fig. 3c). A similar effect of cyclopamine was observed in C2C12 cells grown under the same experimental conditions (data not shown).

We compared the response to Shh of satellite cells isolated from *Ptch1*$^{neo67/+}$ mice with those isolated from wild-type mice. The Shh-induced fold increase in BrdU incorporation was similar in both wild-type and *Ptch1*$^{neo67/+}$ cultures. Interestingly, the basal BrdU incorporation rate (i.e., in the absence of Shh) was significantly higher ($p<0.05$ in the Mann-Whitney U test) in satellite cells from *Ptch1*$^{neo67/+}$ mice than in wild-type cells (fig. 3c).

Next, we investigated the effect of Shh on the differentiation of satellite cells and C2C12 cells to myotubes.

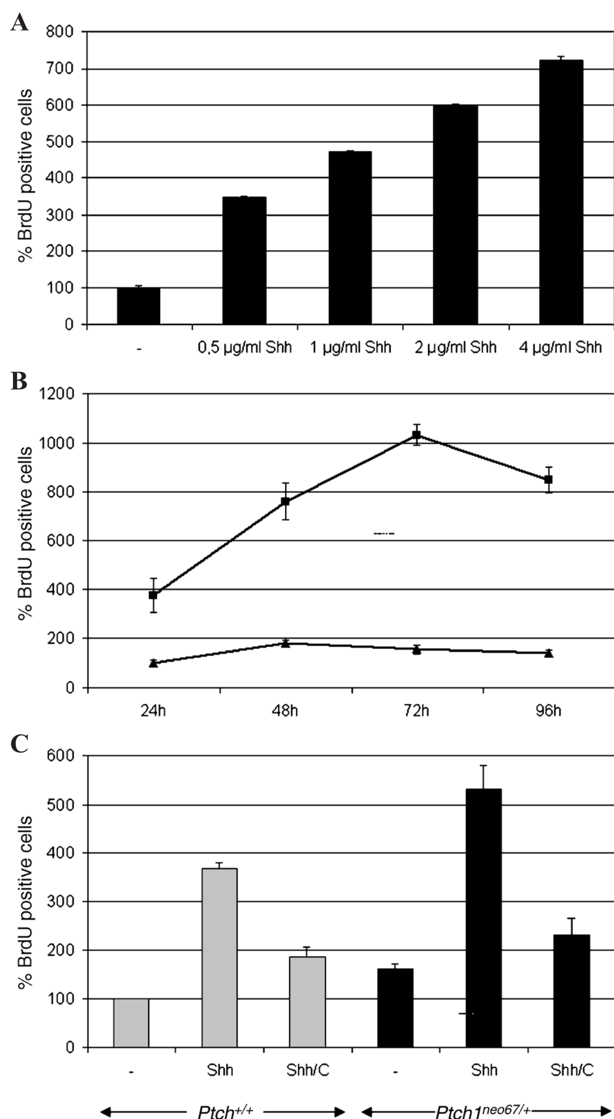

Figure 3. Effects of Shh on proliferation of C2C12 and satellite cells. (*A*) C2C12 cells were cultured for 72 h with the indicated concentrations of Shh-N (R&D). Cells were pulsed with BrdU for the last 24 h and harvested for quantification of incorporated BrdU. (*B*) kinetics of the proliferative response. Untreated and Shh-treated C2C12 (4 µg/ml from R&D) were cultured for the indicated time, pulsed with BrdU and analyzed. (*C*) Satellite cells isolated from wild-type (gray bars) and *Ptch1*$^{neo67/+}$ mice (black bars) were grown for 72 h in differentiation medium with 1 µg/ml Shh in the presence and absence of 1 µM cyclopamine (C). Pulsing with BrdU and analysis was done as described. The results represent the mean value of nine independent experiments.

C2C12 and satellite cells were grown in the respective proliferation medium. At approximately 70% confluency, the medium was switched to the respective differentiation medium (see Materials and methods). After 24 h, Shh was administered in the presence or absence of cyclopamine. Forty-eight hours after treatment with Shh or Shh/cyclopamine, no morphological differences were observed in comparison to untreated cells (fig. 4a–c). The same applied to cells after 72 h (data not shown). In contrast, whereas after 120 h Shh-stimulated cells still retained their mononucleated habitus characteristic of the undifferentiated status (fig. 4e), the untreated and Shh/cyclopamine-treated C2C12 cells differentiated into myotubes (fig. 4d, f). All mononucleated cells and myotubes in untreated (fig. 4g, j) or Shh/cyclopamine-treated (fig. 4i, l) cultures expressed myogenin, a marker of terminal differentiation. In contrast, myogenin-expressing cells were not detected in cultures treated with Shh (fig. 4h, k). These data indicate that Shh specifically blocks differentiation of myogenic precursor cells into myotubes in vitro.

In addition, we investigated the effect of Shh on apoptosis. To this end, TUNEL staining and the expression of the active form of caspase 3 (p17) was determined in cells treated with Shh, with Shh and cyclopamine, and in untreated cells. In C2C12 cells grown under serum-free conditions, the active form of caspase 3 was expressed as early as 72 h in untreated cells and this was accompanied by a low level of TUNEL-positive (i.e., apoptotic) cells (fig. 5a, e). Strikingly, treatment of the cells with Shh inhibited apoptosis and abolished the expression of the active form of caspase 3. This was demonstrated both by immunohistochemistry (fig. 5b, e) and by Western blot (fig. 5j). The inhibitory effects of Shh on apoptosis and caspase 3 activity were reversed by simultaneous treatment of cells with cyclopamine (fig. 5c, f, j). Similarly, Shh inhibited apoptosis and caspase 3 activation in satellite cells and these effects were abolished in the presence of cyclopamine (fig. 5g–i). These data demonstrate an inhibitory effect of Shh on caspase 3 activity. This effect may be responsible for the effects of Shh on apoptosis in myogenic precursors cells.

Discussion

Shh functions as a survival and proliferation factor for myogenic precursor cells during epaxial and hypaxial muscle development [20]. It also represses terminal differentiation of early myogenic precursor cells in hypaxial muscle [21]. This considerable progress in the delineation of Shh function during embryonal myogenesis is in contrast to the knowledge about its relevance in muscle growth and regeneration postnatally.

The identity of the isolated muscle cells as satellite cells was confirmed by the expression of the characteristic markers c-met, *M-cadherin* and *pax7* and by the induction of proliferation in response to HGF [11–12]. Using a variety of techniques, we demonstrated that Shh does exert effects on both primary satellite cells and the related model, C2C12 cells. Both cell systems undergo spontaneous differentiation in culture as evidenced by formation of myotubes and by the expression of myogenin. The effects of Shh on this process are pleiotropic. First, as in

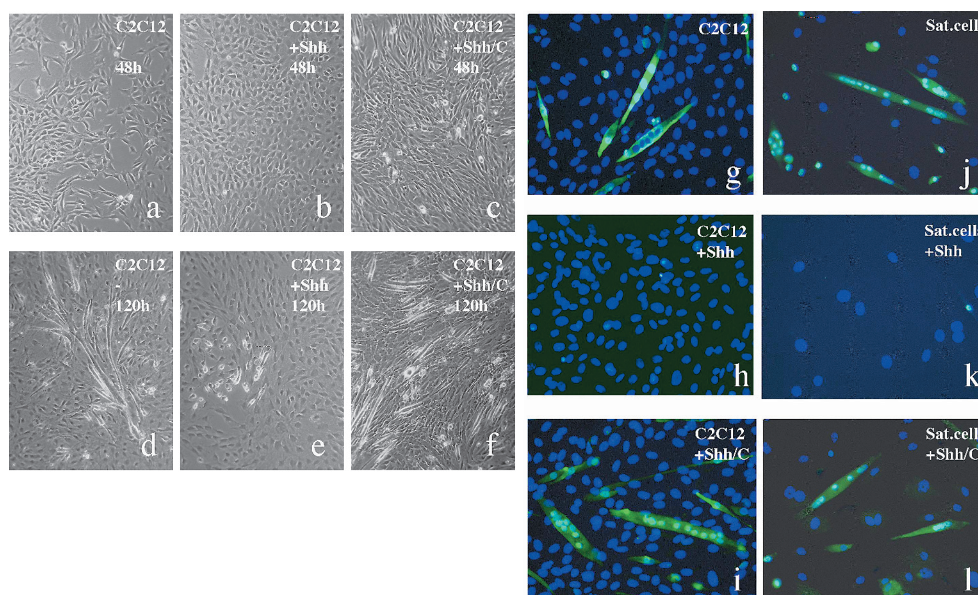

Figure 4. Effects of Shh on differentiation of C2C12 and satellite cells. Cells were grown in differentiation medium with and without Shh. Shh or Shh/C treatment had no effects after 48 h (*a*, *b*, *c*). After 120 h, myotubes had formed in untreated cells but not in those treated with Shh (*d*, *e*). The effect of Shh was reversed by cyclopamine (C) (*f*). Myogenin is expressed in myotubes and mononucleated cells of C2C12 (*g*) and satellite cell (*j*) cultures after 120 h as revealed by immunostaining with a myogenin antibody. Shh effectively blocks myogenin expression (*h*, *k*). This effect is reversed by concomitant treatment with cyclopamine (*i*, *l*).

many other target cells [8], Shh induces the expression of its own signaling pathway, as evidenced by the transcriptional activation of *Ptch1* and *Gli1*. The pathway activation is most likely responsible for the proliferation increase, similar to what has been found in other cell types [22]. Shh can promote its mitogenic effects for long periods of time, since cells do not cease to proliferate over 72 h after a single dose of Shh. Simultaneously, Shh inhibits the differentiation of satellite cells into myotubes. The spontaneous differentiation into myotubes is accompanied by the expression of the characteristic muscle markers such as myogenin. Shh prevents the expression of these markers. The effects of Shh can be reversed by cyclopamine, which is a specific inhibitor of the downstream signaling

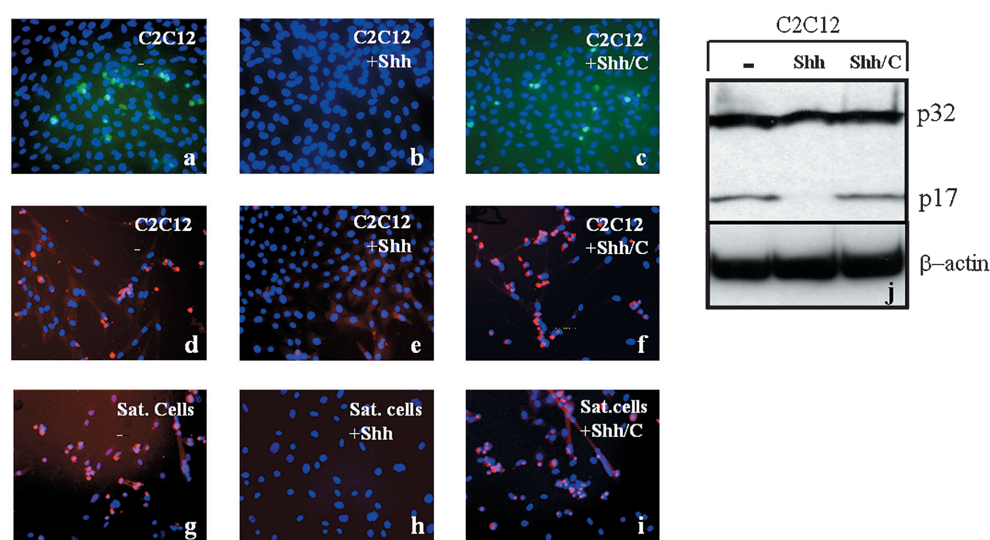

Figure 5. Serum depletion of C2C12 and satellite cells induces apoptosis as revealed by TUNEL- (*a*) and caspase 3-positive cells (*d, g*). In the presence of Shh (*b*), cell death is inhibited. Treatment with Shh blocks expression of the active form of caspase 3, p17 (*e, h, j*), as demonstrated with an antibody against the active form of the protein (*d–j*). The effect of Shh can be blocked by cyclopamine (*c*, *f*, *i*, *j*). p32; caspase 3 precursor protein; C, cyclopamine.

protein Smo. This is direct evidence that Shh acts as a mitogen for myogenic precursor cells, whereas it simultaneously inhibits their differentiation. The data also suggest that satellite cells remain self-renewing precursors as long as they are exposed to Shh.

In line with previous work [23], C2C12 cells undergo apoptosis in the absence of growth factors (fig. 5a). Interestingly, Shh prevents cell death, most likely by blocking caspase 3 activation. This is evidenced by the inhibition of the formation of the active caspase 3 form (p17) by Shh and by the simultaneous decrease in apoptotic cells. The specificity of the Shh effects on caspase 3 and apoptosis is demonstrated by the reversibility of this process by cyclopamine (fig. 5b, j). These results are in agreement with recent data showing that Ptch1 overexpression-induced and caspase-3-mediated apoptosis of 293T and 13.S.24 cells can be reversed by Shh [24].

At a first glance, the differentiation-blocking effect of Shh seems to be opposite to previous experiments in chick myoblast primary cultures and C2C12 cells [25, 26]. In contrast to our experiments, chick primary muscle cells and C2C12 cells treated with Shh exhibited an increase in the number of myotubes. However, the results of all these experiments are not really comparable, due to the very different experimental procedures. Most important, both previous studies utilized Shh-conditioned medium, which contained an unknown concentration of the active form of Shh. Furthermore, C2C12 cells were grown in these studies at an unusually high serum concentration (10%). Finally, the effects of Shh may differ between organisms as different as chick and mouse. On the other hand, the effects of Shh on the proliferation and differentiation of myogenic precursor cells resemble those previously reported for HGF. HGF enhances proliferation of satellite cells and inhibits their differentiation, the latter evidenced by the suppression of the activity/expression of myogenin [27]. In our experiments the proliferation rate of the cells induced by Shh is comparable to that following HGF exposure, which is an established mitogen in satellite cells [11, 12]. This additionally demonstrates the validity of the cell systems used as models of myogenic precursors.

The described effects of Shh on proliferation, differentiation, and apoptosis of muscle satellite cells *in vitro* may reflect the role of Shh during skeletal muscle growth, regeneration, and tumorigenesis *in vivo*. With regard to muscle growth, muscle fibers ectopically expressing Shh due to injection of an Shh-expressing plasmid are 47% larger than control-injected muscle fibers [28]. Whether Shh is activated during muscle injury is not known. However, during injury repair in tissues such as epithelium of the lung and gut, Shh is required and exerts a strong proliferative stimulus on progenitor cells [3, 29, 30]. The inflammation- or injury-damaged progenitor cells might accumulate oncogenic mutations at an increased rate [4]. Activation of Shh signaling may thus lead to uncontrolled growth of these cells, in extreme cases resulting in tumor formation. In agreement with this hypothesis, Shh has been demonstrated to act on precursor or stem cells in an increasing number of tissues, possibly contributing to the development of several common cancers, such as prostate, MB, BCC, GI, and lung cancers [2–6]. A wide range of these tumors displayed increased Shh pathway activity as revealed by *Gli1* and *Ptch1* expression, and growth of these tumors has been shown to be Shh-dependent. Abnormal activation of the Shh pathway also leads to the development of muscle tumors. RMS, which develops in *Ptch1* heterozygotes, overexpresses *Ptch1* and *Gli1*. This together with our observations that *Ptch1* and *Gli1* can be induced in satellite cells upon Shh stimulation (fig. 2) and that Shh induces growth and inhibits differentiation and apoptosis of these cells, supports the hypothesis that RMS is derived from satellite cells [10].

Together, our data provide new insights into the molecular mechanisms of regulation of satellite cell growth and differentiation. The introduction of Shh as an important growth factor for satellite cells might impact on the progress in therapies of muscle-wasting diseases in the future. It also provides new aspects of tumorigenesis of the striated muscle and supports the hypothesis that RMS arises from satellite cells [10].

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