Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation

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SUMMARY

The proteins of the hedgehog (Hh) family increase the osteoblastic output of BMPs via Smad signaling. Furthermore, N-Shh was found to abolish adipocytic differentiation of C3H10T1/2 cells both in the presence or absence of BMP-2. A short treatment with N-Shh was sufficient to dramatically reduce the levels of the adipocytic-related transcription factors C/EBPα and PPARγ in both C3H10T1/2 and calvaria cell cultures. Given the inverse relationship between marrow adipocytes and osteoblasts, agonists of the Hh signaling pathway might constitute potential drugs for preventing and/or treating osteopenic disorders.

Key words: Sonic hedgehog, Bone morphogenetic protein 2, Osteoblast, Adipocyte, Differentiation

INTRODUCTION

The proteins of the hedgehog (Hh) family mediate a central role in the control of pattern formation and cellular proliferation during development (Hammerschmidt et al., 1997; Perrimon, 1995). In vertebrates, the hedgehog family consists of at least three members: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Fietz et al., 1994). Autocatalytic processing mediated by the C-terminal domain of the Hh precursor protein generates an N-terminal product that accounts for all known signaling activity (Lee et al., 1994). Biochemical and genetic data suggest that the hedgehog receptor is the product of the tumor suppressor gene, patched (Ptch) (Marigo et al., 1996; Stone et al., 1996), and that a distinct membrane protein, smoothened (Smo), functions as the signaling component of the Shh receptor (Murone et al., 1999). Two different Ptch receptors (Ptch1 and Ptch2) have been characterized in vertebrates (Carpenter et al., 1998). In the absence of Hh induction, the activity of Smo is inhibited by Ptch probably through their physical association (Ingham, 1998). Members of the Ci/Gli family of DNA-binding proteins are the major downstream transcriptional effectors that mediate Hh signaling in animal cells (Ruiz i Altaba, 1997).

Recently, members of the hedgehog gene family have been shown to regulate skeletal formation in vertebrates. Although Ihh was first identified as a regulator of chondrocyte differentiation (Vortkamp et al., 1996), Ihh signaling has been shown to be essential for normal osteoblast development in endochondral bones (St-Jacques et al., 1999). Mice in which Shh has been deleted fail to form vertebrae and display severe defects of distal limb skeletal elements (Chiang et al., 1996). Shh seems to affect both chondrocyte and osteoblast differentiation. Overexpression of Shh and in vitro chondrogenic cultures promoted characteristics of hypertrophic chondrocytes (Stott and Chuong, 1997) and Shh has been shown to mediate the survival of both myogenic and chondrogenic cell lineages in the somites (Teillet et al., 1996).}

In vitro, Shh has been demonstrated to induce alkaline phosphatase (ALP), a marker of osteoblast differentiation, in the mouse mesenchymal cell line C3H10T1/2 (Katsuura et al., 1999; Kinto et al., 1997; Murone et al., 1999; Nakamura et al., 1997) and the osteoblast cell line MC3T3-E1 (Nakamura et al., 1997). Interestingly, intramuscular transplantation of fibroblasts expressing Shh into athymic mice induced ectopic bone formation (Kinto et al., 1997).
In the present study, we analyzed the effects of Shh on osteoblastic and adipocytic differentiation by using either pluripotent cell lines or murine calvaria cultures. Interestingly, we found that Shh increases the commitment of mesenchymal cell lines and calvaria cells to the osteoblastic lineage in response to BMP-2. Shh also inhibits the ability of these cells to differentiate into adipocytes. Given that the bone loss occurring with aging is associated with reduced osteoblastic bone formation and an increased volume of marrow adipose tissue, the Shh pathway might constitute a good therapeutic target to treat osteopoenic disorders.

**MATERIALS AND METHODS**

**N-terminal Shh production**

The N-terminal part of the murine Shh (corresponding to amino acids 25-198) was isolated by RT-PCR and confirmed by nucleotide sequencing. The isolated sequence was subcloned into the mammalian expression vector pABWN under control of CMV enhancer/chicken β-actin promoter (pShh). pShh was transfected into mouse fibroblast L-cells (LTK-P2 cells) and stably transfected cells were established by selection with G148 (800 µg/ml) medium. N-Shh was purified from cell culture medium as follows. The pH of the cellular culture medium was adjusted to 7.5 by adding Tris buffer (50 mM final concentration). DTT (0.5 mM) and PMSF (0.5 mM) were added to the medium before its clarification by centrifugation at 42,000 g for 3 hours at 4°C. The conductivity of the supernatant was adjusted to 14 mS by adding NaCl then loading on a 25 ml SP Sepharose Fast Flow resin column (Pharmacia). The column was pre-equilibrated with buffer A (25 mM Tris, pH 7.5, 120 mM NaCl and 0.1 mM PMSF). Elution was developed with a linear gradient to 60% buffer B (buffer A containing 1 M NaCl). Fractions were analyzed for Shh content by SDS-PAGE, followed by coomassie-blue staining.

Shh was then concentrated on a 5 ml Hitrap SP column (Pharmacia) and further purified using a gel filtration column (Superdex 200 pg, Pharmacia). Final fractions containing Shh were pooled then stored at −80°C until used.

**Cell culture**

C3H10T1/2 (obtained from ATCC) and MC3T3-E1 (kindly provided by R. Francesch) cell lines were cultured (5% CO2 at 37°C) in αMEM supplemented with 10% heat inactivated fetal calf serum. C2C12 cells (kindly provided by G. Karlsen) was (5% CO2 at 37°C) in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum. For treatment or transient transfection, cells were plated at 2×10^4/cm^2 and 24 hours later the culture medium was changed for that with 2% fetal calf serum. Treatment or transfections were carried out as indicated below.

**Calvaria cell preparation**

Murine calvaria cells were obtained from the calvariae of neonatal mice 1-2 days after birth by sequential collagenase digestion at 37°C. Calvariae were removed from the animals under aseptic conditions and incubated at 37°C in DMEM containing trypsin (0.5 mg/ml) and EDTA (1.5 mg/ml) under continuous agitation. Trypsin digest were discarded at 15 minutes and replaced with DMEM containing 1 mg/ml of collagenase. The collagenase digest were discarded at 20 minutes and replaced with fresh enzyme dilution. The cells released between 20-40 minutes were collected by a short passive sedimentation step, and two centrifugation steps (400 g, 10 minutes) and cultured in proliferation medium (DMEM supplemented with 20% FCS and 2 mM glutamine) at a density of 2.5×10^5 cells per cm^2 in Petri dishes (100 mm diameter). Calvaria cells were cultured until 80% confluence and stocks were frozen. For the experiments described here, cells were thawed in proliferation medium; two days later, this medium was replaced by differentiation medium (αMEM containing 10% FCS, 2 mM glutamine, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate) and stimulated with the different agents for the indicated times.

**Measurement of alkaline phosphatase activity**

Cells were treated for the indicated time with BMP-2, Shh or BMP-2/Shh, and alkaline phosphatase (ALP) activity was determined in cell lysates using Alkaline Phosphatase Opt kit (Roche Molecular Biochemicals). Cell lysates were analyzed for protein content using a micro-BCA Assay kit (Pierce), and ALP activity was normalized for total protein concentration. For histochemical analysis of plasma-membrane-associated ALP, after stimulation with BMP-2, Shh or BMP-2/Shh for the time indicated, cells were washed three times with PBS and stained using Alkaline Phosphatase Leukocyte Staining Kit (Sigma, St Louis, MO), according to the manufacturer's protocol.

**Plasmids, cell transfection and assay for luciferase activity**

The gal4-Smad1 construct was provided by A. Atfi. The pAl promoter luciferase construct (paP2/luc) was made by subcloning the 5.4 kb KpnI-Smal insert isolated from pBluescript KS+ into the pGL3-basic vector (Promega). Gli1 and Noggin were isolated by RT-PCR and clones were confirmed by DNA sequence analysis. Gli1 and Noggin were subcloned into pcDNA3.1 vector (Invitrogen). pRSV-PK1 and pRSV-PKmut were kindly provided by R. A. Maurer.

C3H10T1/2 cells plated in 24-well plates, as indicated above, were transiently transfected with the indicated construct (1 µg) using DNA-lipid complex Fugene 6 (Boehringer Mannheim) according to the manufacturer's protocol. To assess transfection efficacy 20 ng of pRL-TK (Promega, Madison, WI), which encodes a Renilla luciferase gene downstream of a minimal HSV-TK promoter, was systematically added to the transfection mix. In experiments using pGli1 and pNog constructs, controls were carried out by replacing constructs with empty pcDNA3. 16 hours after transfection, cells were washed, cultured in medium at 2% fetal calf serum and either left unstimulated or stimulated with Shh for an additional 48 hours. ALP activity was determined in cell lysates as indicated above. When luciferase reporter constructs were used, luciferase assays were performed with the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. 10 µl of cell lysate was assayed first for firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

**RNA purification and gene expression analysis by real-time TaqMan PCR**

Cells were treated as indicated above and total RNA was isolated from cultured cells using total isolation kit from Quantum Appligene (Ilkirch, France). ALP, PPARγ, C/EBP1α, p2 and leptin mRNA expression was determined by RT followed by real-time TaqMan PCR analysis. Optimal oligonucleotide primers and TaqMan probes (Table 1) were designed using Primer Express V1.0 (Perkin-Elmer Applied Biosystems Inc.) using murine ALP (Accession J02980), PPARγ, C/EBP1α, p2 and leptin sequences from the GenBank database. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers and TaqMan probe (labeled with Vic fluorochrome) were from Perkin-Elmer Applied Biosystems.

RT reactions were carried out using 1 µg total RNA at the following conditions: 42°C for 60 minutes, 95°C for 5 minutes, 4°C for 5 minutes. RT product were diluted three times in sterile bi-distilled water and 5 µl were used to perform TaqMan PCR. ALP TaqMan PCR was carried in a final volume of 25 µl containing: 1X TaqMan EZ buffer, 5 mM Mn(Oac)2, 200 µM dA/dC/dG/dUTP, 0.625 unit AmpliTaq Gold, 300 nM of each of mALP1 and mALP1 (forward and reverse), 40 nM of each of GAPDH reverse and forward primers.
and 200 nM of each of GAPDH and ALP TaqMan probes. Cycling conditions were 95°C for 15 seconds, 60°C for 1 minute for 40 cycles. Real-time TaqMan PCR was performed in ABI PRISM 7700 Sequence detector (Perkin-Elmer Applied Biosystems Inc.). Conditions for PPARγ, C/EBPα, aP2 and leptin TaqMan PCR were exactly the same as for the ALP reaction, except that ALP primers and probe were replaced by PPARγ, C/EBPα, aP2 or leptin ones. All PCR reactions were performed in duplicate and ALP, PPARγ, C/EBPα, aP2 and leptin signals were normalized to GAPDH signal in the same reaction.

**Determination of triglyceride**

C3H10T1/2 cells were cultured as indicated above, then either left unstimulated or stimulated with Shh. The accumulation of intracellular triglyceride droplets was visualized by staining with Oil Red O. Triglyceride release into culture supernatant was measured using Sigma diagnosis glycerol-triglyceride (GPO-Trinder) kit according to the manufacturer’s specification (Sigma).

**RESULTS**

Shh has been reported to induce alkaline phosphatase (ALP) activity in the murine mesenchymal pluripotent cell line C3H10T1/2 (Katsuura et al., 1999; Kinto et al., 1997; Murone et al., 1999; Nakamura et al., 1997). To further investigate the effect of Shh on the osteoblast commitment we studied the effect of recombinant murine N-terminal Shh (N-Shh) on the ALP activity displayed by three mesenchymal murine cell lines (C3H10T1/2, ST2 and C2C12) that can differentiate into the osteoblastic lineage in the presence of bone morphogenetic factors.

**Table 1. Primer and probe sequences used for TaqMan PCR analysis**

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Probe (5'-3)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>TCAGGCCATGAGGCTCAGAT</td>
<td>CACATGCCAGGCTGCTCCA</td>
<td>AGCATCTCTGCCCAGAG</td>
</tr>
<tr>
<td>Leptin</td>
<td>GTCTTTGTGCTGAAGTAGAAGG</td>
<td>CGCCGGAGAGTGGTCATAA</td>
<td>TCACACATACATATAAAATCGAGGCTAGTC</td>
</tr>
<tr>
<td>aP2</td>
<td>GTCCAATCCGGTCAGAGATTAC</td>
<td>TGGTCTGTGGGTATTTCATC</td>
<td>CGAGATTTCCTCAAATGCGGTGG</td>
</tr>
<tr>
<td>PPARγ</td>
<td>TGAACACTGTGGAGATTCTCTG</td>
<td>CCAATTGAATTCTCTTTGAGTC</td>
<td>CCAGAGCAGATGGGCTCTCCTG</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>GACAAGAAACAGCAACGAGTC</td>
<td>GCGGGTCATGTCTCAGGTTC</td>
<td>AACACACATGCGGTTGCCAGAG</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>CACCCCGGGGACAGGTT</td>
<td>CTAATAATGCTGGACGCTCTCTG</td>
<td>CAGACAGATGGGCTCTCAGAGCCT</td>
</tr>
<tr>
<td>Osf2/Cbfa1</td>
<td>TTATGGGCSCATTCTCTATC</td>
<td>TGCTCTGTGAGGTCAGATCGT</td>
<td>AGAACCACCGGCCCTCCTGAAC</td>
</tr>
</tbody>
</table>

*Labeled with FAM fluorochrome.*
protein 2 (BMP-2), and the already committed immature osteoblastic cell line MC3T3-E1. As shown in Fig. 1, in the absence of BMP-2 only C3H10T1/2 cells responded significantly to N-Shh with an increase in ALP activity. Whereas N-Shh did not significantly modify the ALP activity levels induced by different doses of BMP-2 in MC3T3-E1 and C2C12 cells, N-Shh and BMP-2 displayed a synergistic effect on the stimulation of ALP activity in both ST2 and C3H10T1/2 cells (Fig. 1). To determine whether this was the result of an increase in the expression of ALP by individual cells or of an increase in the number of cells expressing the enzyme, we used an enzymatic staining assay to investigate whether N-Shh could modify the percentage of cells displaying ALP activity in response to BMP-2. Interestingly, N-Shh induced a significant increase in the percentage of cells responding to BMP-2 in terms of ALP activity in both C3H10T1/2 (Fig. 2) and ST-2 (data not shown).

Given that C3H10T1/2 were the only cells responding to N-Shh in the absence of BMP-2, we evaluated the capacity of N-Shh to affect the expression of other osteoblast markers, including the transcription factor Cbfa1/Osf2 and the selective mature osteoblast marker osteocalcin (OC). For this purpose, C3H10T1/2 cells were transfected with a plasmid encoding N-Shh; 5 days later, RNA was extracted and the expression of these two genes was determined by real-time TaqMan PCR. As shown in Fig. 3, as expected, N-Shh significantly upregulated the gene expression of ALP (20-fold), but also induced the expression of Cbfa1/Osf2 (2.5-fold) and OC (5-fold). These data strongly suggest that N-Shh increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage.

We then investigated whether spontaneous or N-Shh-induced BMP secretion by these cells could account for the effects of N-Shh on ALP activity. It has been reported that N-Shh treatment does not affect the expression levels of BMP-2, -4, -5, -6 and -7 genes in C3H10T1/2 cells (Nakamura et al., 1997). In our hands, BMP-2 RNA expression level was unmodified in the presence of N-Shh, as assessed by TaqMan quantitative PCR (data not shown). Furthermore, we carried out experiments in which BMP signaling was abolished by transiently transfecting cells with an expression vector encoding the BMP inhibitor Noggin (Zimmerman et al., 1996). Under our experimental conditions, transfection with Noggin abolished the ability of BMP-2 to induce ALP activity (Fig. 4A). Noggin was effective over a broad range of BMP-2 concentrations, from 100 to 1000 ng/ml (data not shown). Transfection of cells with a plasmid encoding N-Shh, like recombinant N-Shh, induced an important increase in ALP activity in C3H10T1/2 cells (Fig. 4B). Interestingly, co-transfection of these cells with N-Shh and Noggin did not significantly modify the Shh-induced ALP activity (Fig. 4B). Thus, N-Shh can induce ALP in C3H10T1/2 cells even in the absence of BMPs. Transfection of C3H10T1/2 cells with a plasmid expressing Gli1, a transcription factor identified as a candidate downstream mediator of the Shh response (Dominguez et al., 1996), also induced ALP activity (Fig. 4C) indicating that Gli-1 overexpression mimics the effect of N-Shh. Furthermore, Noggin overexpression had no effect on Gli-1-induced ALP activity (Fig. 4C). We conclude from these experiments that the stimulatory effect of N-Shh on osteoblastic differentiation is direct and independent of the autocrine stimulation by BMPs.

The observed synergistic effects of N-Shh and BMP-2 led us to conduct experiments in which cells were pretreated with N-Shh for different periods of time, before stimulating C3H10T1/2 cells with BMP-2 to determine how long N-Shh had to be present to enhance the BMP-induced ALP activity. As shown in Fig. 5, N-Shh pretreatment induced a significant and time-dependent increase in ALP activity in response to BMP-2. The synergy was maximal between 1 and 6 hours of N-Shh pretreatment (data not shown). When BMP-2 was added 24 hours after the N-Shh pulse, no increase in ALP activity

![Fig. 2. Shh increases the number of alkaline phosphatase-positive cells in response to BMP-2. C3H10T1/2 cells were stimulated with either BMP-2 (100 ng/ml) or BMP-2 and Shh (10 μg/ml).](https://example.com/figure2.png)

(A) After three days, cells were stained for plasma-membrane-associated ALP as indicated in Materials and Methods. (B) The percentage of ALP-positive cells was determined from six series of treatment. Results are expressed as mean±s.d. Statistically significant changes were detected using Student’s t-test (*P<0.001).
adipocytic differentiation, we used real-time quantitative PCR to examine the expression of a series of genes related to the adipocyte commitment, including C/EBPα, PPARγ2, adipocyte fatty acid binding protein (aP2) and leptin. As illustrated in Fig. 8A, the expression of aP2 and leptin was significantly reduced by the treatment with N-Shh. In addition, N-Shh dramatically decreased the expression of C/EBPα and PPARγ2 (Fig. 8A), two transcription factors that play a crucial role in the adipocyte differentiation program that induces the maturation of pre-adipocytes into fat cells (Rosen et al., 1999; Rosen et al., 2000; Wu et al., 1999). Not surprisingly, in the absence or presence of BMP-2, N-Shh reduced dramatically the number of adipocytic cells in these cultures, as assessed by Oil Red O staining (Fig. 7). Triglyceride levels in cell supernatants (indicative of lipase activity) were also dramatically reduced in N-Shh-treated cells (data not shown).

To further investigate the inhibitory effect of Shh on adipocytic differentiation, we used real-time quantitative PCR to examine the expression of a series of genes related to the adipocyte commitment, including C/EBPα, PPARγ2, adipocyte fatty acid binding protein (aP2) and leptin. As illustrated in Fig. 8A, the expression of aP2 and leptin was significantly reduced by the treatment with N-Shh. In addition, N-Shh dramatically decreased the expression of C/EBPα and PPARγ2 (Fig. 8A), two transcription factors that play a crucial role in the adipocyte differentiation program that induces the maturation of pre-adipocytes into fat cells (Rosen et al., 1999; Rosen et al., 2000; Wu et al., 1999). Not surprisingly, in the absence or presence of BMP-2, N-Shh reduced dramatically the number of adipocytic cells in these cultures, as assessed by Oil Red O staining (Fig. 7). Triglyceride levels in cell supernatants (indicative of lipase activity) were also dramatically reduced in N-Shh-treated cells (data not shown).

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Shh

experiment, pGli1 was replaced by empty vector (pcDNA3). Transfected cells were cultured for 24 hours in the absence or presence of BMP-2. Renilla activity obtained from pTK-RL plasmid. (B) C3H10T1/2 were co-transfected with Gal4-Smad1, pGli1 and pG15E1b-luc. In the control was evaluated and normalized to (mean luciferase fold induction±s.d.) are representative of two independent experiments, each performed in triplicate.

same conditions, ALP gene expression was found to be dramatically increased (Fig. 8A). Pulse experiments were then performed to evaluate how long Shh had to be present to inhibit the adipocyte commitment of C3H10T1/2 cells. As shown in Fig. 8B, pre-treatment with N-Shh for 1 hour was sufficient to significantly reduce adipocytic commitment as assessed by measuring the gene expression of the adipocyte markers C/EBPα and PPARγ2, and aP2 and leptin. It is interesting to note that a 1 hour pulse of N-Shh was not sufficient to reach the strong increase in ALP mRNA levels found when N-Shh was not removed from the medium (Fig. 8A,B).

We then determined whether transfecting cells with the transcription factor Gli1 could mimic the inhibitory effect of Shh on adipocytic differentiation. For this purpose, we took advantage of the fact that N-Shh significantly decreases luciferase activity in cells transfected with a construct in which this reporter gene was under the control of an aP2 promoter (Fig. 9). Interestingly, co-transfection of C3H10T1/2 cells with Gli1 resulted in a significant reduction of luciferase activity (Fig. 8), strongly suggesting that Gli1 plays a crucial role in the ability of N-Shh to inhibit adipogenesis in these cells.

Given that Ihh has been shown to induce PTHrP in the periarticular perichondrium (Vortkamp et al., 1996), and that it has been recently reported that PTHrP abolishes adipocytic differentiation in both pre-adipocyte 3T3-L1 and C3H10T1/2 cell lines (Chan et al., 1999; Chan et al., 2000), we investigated whether N-Shh induces PTHrP in C3H10T1/2 cells. As assessed by real-time PCR, no increase in PTHrP gene expression could be demonstrated in cells treated for different periods of time with N-Shh. Moreover, PTHrP protein was not detected by radioimmunoassay in both untreated and N-Shh-treated cells. Finally, whereas BMP-2 significantly induced the expression of PTH/PTHrP receptor in C3H10T1/2 cells, N-Shh did not significantly modify the expression of this receptor both in the presence or absence of BMP-2 (data not shown). These data suggest that the mechanism by which N-Shh inhibits adipogenesis is not related to an autocrine loop involving PTHrP.

Together, our results indicate that N-Shh differentially regulates the osteoblastic and adipocytic commitment in a pluripotent cell line. It was therefore very interesting to investigate whether comparable effects are found using primary cells in culture. Calvaria cells constitute a good cellular model because these cells differentiate into osteoblasts in the presence of serum, ascorbate and β-glycerolphosphate. Moreover, in these conditions, adipogenesis can also be observed. In fact, most of the adipocyte markers are induced during the culture (data not shown). Mouse calvaria cells prepared as described in Materials and Methods were cultured in the presence of N-Shh, BMP-2 or both. After 5 days of culture, ALP activity was measured and RNA expression levels of aP2, PPARγ2 and C/EBPα were determined and compared with control unstimulated cells. As shown in Fig. 10A, N-Shh alone did not affect the ALP activity of calvaria cells but it significantly increased the ALP activity displayed by calvaria cells at day 5 of culture in the presence of BMP-2. N-Shh alone dramatically reduced the adipocytic markers in calvaria cells (Fig. 10B).

Fig. 6. Shh enhances BMP-2 mediated Smad1 activation. (A) C3H10T1/2 or ST2 cells were transiently cotransfected, as indicated in Materials and Methods, with an expression vector coding for Smad1 fused to the yeast Gal4 binding site (pGal4-Smad1) and a reporter plasmid driven by the Gal4 binding site (pG15E1b-luc). To normalize luciferase signal, pTK-RL was included in the transfection mix. Transfected cells were either left unstimulated (CTRL) or stimulated with BMP-2 (100 ng/ml) and Shh (10 μg/ml) for 24 hours. Cells were then lysed and luciferase activity was evaluated and normalized to Renilla activity obtained from pTK-RL plasmid. (B) C3H10T1/2 were co-transfected with Gal4-Smad1, pGli1 and pG15E1b-luc. In the control experiment, pGli1 was replaced by empty vector (pcDNA3). Transfected cells were cultured for 24 hours in the absence or presence of BMP-2 (100 ng/ml). Luciferase activity was determined in cell lysates and normalized to Renilla activity obtained from pTK-RL plasmid. Results (mean luciferase fold induction±s.d.) are representative of two independent experiments, each performed in triplicate.

Fig. 5. A short time of Shh treatment is sufficient to enhance BMP-2 activity. C3H10T1/2 cells were treated for the time indicated with Shh (10 μg/ml) then washed with sterile PBS and cultured in the presence of BMP-2 (100 ng/ml) for additional 5 days. ALP activity was measured in cell lysates and normalized to protein content. Results (mean±s.d.) are representative of three independent experiments, each performed in triplicate.
DISCUSSION

Members of the Hh gene family were initially characterized as patterning factors in embryonic development, but recently they have also been shown to regulate skeletal formation in vertebrates (reviewed by Iwamoto et al., 1999). The induction of ectopic cartilage and bone formation by N-Shh (Kinto et al., 1997) suggests that Hh proteins act on skeletal cells, including mesenchymal progenitor cells, chondrocytes and osteoblasts. The mesenchymal cell line C3H10T1/2, which possesses the ability to differentiate in the presence of BMP-2 into different mesenchymal lineages including osteoblasts (Ahrens et al., 1993; Wang et al., 1993), has been demonstrated to respond to Shh modulation of osteoblastic and adipocytic commitment.
Shh by increasing the expression of the osteoblast marker ALP (Katsuura et al., 1999; Kinto et al., 1997; Murone et al., 1999; Nakamura et al., 1997). Only a fraction of C3H10T1/2 cells are capable of acquiring an osteoblast phenotype when stimulated with BMP-2. We have shown here that N-Shh alters the ability of C3H10T1/2 cells, as well as the stromal cell line ST2, to respond to BMP-2 in terms of osteoblastic commitment. Moreover, our data demonstrate that N-Shh alone induces osteoblast commitment and blocking adipocytic differentiation of C3H10T1/2 cells. In calvaria cells, a synergy between BMP-2 and N-Shh concerning osteoblastic differentiation and an inhibition of adipocytic commitment was demonstrated.

One of the most interesting observations of this study is the synergistic effect of BMP-2 and N-Shh on the osteoblast differentiation. Although BMP-2 was shown to be dispensable for a slight induction of ALP by N-Shh in C3H10T1/2 cells, cooperation between BMP-2 and N-Shh was necessary to promote a strong induction of the osteoblast marker. This synergy might be explained by a modulation exerted by N-Shh on the signaling by BMP-2 or vice-versa. Currently, the intracellular signals induced by Hh proteins in mammal cells are poorly understood. Smo has been demonstrated to be the signaling component of the Hh receptor complex, whereas patched is considered as a ligand-regulated inhibitor of Smo (Murone et al., 1999). Smo activity requires the third intracellular loop of Smo, a domain typically involved in the coupling of seven transmembrane receptors to G protein effectors. However, so far there is no evidence that second messengers implicated in G-protein-coupled receptor signalling take part in the Hh response (Murone et al., 1999). Members of the Ci/Gli family of DNA-binding proteins are the major downstream transcriptional effectors mediating Hh signaling (Ruiz i Altaba, 1997). Studies conducted in Drosophila have shown that, in the absence of Hh, full-length Ci, which is localized in the cytoplasm, is processed into an N-terminal nuclear repressor form. Phosphorylation of Ci by PKA may target this protein for ubiquitination and processing by the 26S proteasome. In the presence of Hh, phosphorylation of Ci is suppressed, possibly a consequence of inhibiting PKA (reviewed by Ingham, 1998). Concerning BMP signaling, it is well established that the main intracellular signaling mediators are the different members of the Smad protein family. Receptor-regulated Smad1, Smad5 and Smad8 are the targets of BMP receptors. After phosphorylation, receptor-regulated Smads associate with the common Smad, Smad4, and the heteromeric complex is translocated into the nucleus where it activates specific genes through cooperative interactions with DNA and other DNA-binding proteins such as FAST1, FAST2 and Fos/Jun (reviewed by Derynck et al., 1998). Recently, two examples of crosstalking between BMP and Hh signaling have
been reported. First, truncated Gli3 proteins have been demonstrated to associate with Smads (Liu et al., 1998). Second, Shh has been shown to promote somitic chondrogenesis by altering the cellular response to BMP signaling (Murtaugh et al., 1999).

Concerning the synergy between BMP-2 and N-Shh described here, we have demonstrated that a short pre-treatment (30-60 minutes) with N-Shh followed by BMP-2 stimulation is sufficient to obtain the synergy between these proteins. Interestingly, no synergy was demonstrated with a longer pre-treatment with Shh (24 hours). This suggests that Shh provides a transient window of competence during which time BMP signals induce osteoblastic differentiation in cells otherwise refractory to osteoblast commitment in response to BMP-2. A similar mechanism has been recently proposed to explain the effects of Shh in the promotion of somitic chondrogenesis (Murtaugh et al., 1999). Shh can alter BMP responsiveness by directly affecting one or several proteins in the cascade of BMP signaling events. We have demonstrated that the synergistic N-Shh/BMP-2 effect is in part mediated by an N-Shh modulation of the BMP signaling pathway component Smad1. Indeed, N-Shh increases BMP-2-mediated Smad1 transcriptional activity in both C3H10T1/2 and ST2 cells. In addition, this modulation seems to be independent of the well known Hh pathway transcription factor Gli1, because transfection with Gli1 did not alter the transcriptional activity of Smad1 (Fig. 6), suggesting that Hh signaling components upstream of Gli are integrated with the BMP signaling complex to affect Smad activity. The enhancement of Smad1 transcriptional activity may be the result of a positive effect in the life span/availability, phosphorylation or nuclear accumulation of Smad1 or an indirect effect on a co-activator or co-repressor involved in the transcriptional machinery of Smad1. We are currently investigating whether N-Shh modifies the expression of a series of proteins described as capable of interacting with BMP signaling.

Another interesting finding described in this study is the fact that the ability to respond to N-Shh in terms of ALP expression seems to be dependent on the stage of differentiation of cells. N-Shh displayed synergistic effect on stromal cell line ST2 (described as a pre-adipocyte cell line but able to differentiate into an osteoblast in the presence of BMP-2) and mesenchymal pluripotent C3H10T1/2 cells. On the contrary, the pre-osteoblastic MC3T3-E1 cells and the mature osteoblastic cell lines ROS 17/2.8 and ROB-C26 (data not shown) are insensitive to N-Shh in terms of ALP induction in the presence of BMP-2. This suggests that only immature pluripotent cells respond to N-Shh. A synergistic effect of N-Shh and BMP-2 was clearly demonstrated in calvaria, in which there is a heterogeneous population including cells at different stage of differentiation.

It is important to point out that, in the absence of BMP-2, only C3H10T1/2 was shown to significantly respond to N-Shh in terms of ALP expression. This response could be mimicked by transfecting cells with a Gli1 expression vector, thus suggesting that the activity is dependent on the activity of this transcription factor. Our results suggest that there are at least two mechanisms by which Shh regulates osteogenic differentiation of cells: directly via a Gli-dependent manner and indirectly via a Gli-independent modulation of BMP signaling via Smad, as discussed above. In addition, our results suggest that these two mechanisms are triggered at the same time only in cells displaying a particular stage of differentiation, because only the more immature cells studied here, C3H10T1/2, respond to N-Shh alone. Although N-Shh alone did not affect the osteoblastic commitment in calvaria cells, one could not exclude the existence of a subpopulation of calvaria cells that respond to N-Shh in the absence of BMP-2 but, if they exist, the fraction of responding cells is very small.

Hh molecules have been shown to control the differentiation of different cell types. Here, we present evidence that N-Shh dramatically compromises the adipocytic commitment of both the pluripotent mesenchymal cell line C3H10T1/2 and calvaria cells. The decrease in the number of mature adipocytes was evaluated by looking at the presence of lipidic vacuoles in cells, by measuring the lipase activity and also by looking at the gene expression of a number of adipocytic markers. Thus, Shh dramatically reduced the levels of PPARγ and C/EBPα, two transcription factors playing a central role in adipogenesis. Furthermore, we have shown that overexpression of the transcription factor Gli1 in the cells mimics this activity. How Gli1 can affect adipocytic differentiation needs to be explored in detail. Gli1 has not been described as directly repressing any gene transcription, therefore one possibility is that Shh via Gli1 induces the expression of molecules that inhibit adipocytic commitment. One candidate could be PTHrP, which can be induced in mesenchymal cells and osteoblasts and has been described as negatively affecting adipogenesis. However, our data demonstrate that PTHrP is not involved in the effects described here. We are currently using genome-wide expression analysis to study the regulation of genes by N-Shh in C3H10T1/2 to select candidate genes potentially involved in the activity of N-Shh on adipocytic differentiation.

The potential in vivo relevance of our results remains to be elucidated. Concerning the expression of Hh proteins in vivo, Shh signal is observed in the posterior mesoderm at the initial stage of limb development, and remains distant from the area where skeletal elements appear. By contrast, the distribution of Ihh signals is closely related to cartilage-forming regions. Little information exists about the expression of hedgehog molecules in osteoblastic or adipocytic lineages in adult tissues, but the expression of Shh in cells surrounding the peristium after fracture has recently been reported in mice (Kuriyama et al., 2000). None of the different cell lines used in our study expresses Shh mRNA (data not shown). These data suggest that the source of Hh proteins in vivo must be other than the osteoblasts or their precursors. Concerning the effect of Shh on adipocytic commitment, no relevant finding has been described in mice lacking Shh gene function (Chiang et al., 1996).

Total marrow fat increases with age, and there is an inverse relationship between marrow adipocytes and osteoblasts with aging (Beresford et al., 1992; Burkhardt et al., 1987). The number of mesenchymal stem cells with osteogenic potential decreases early during aging in humans and may be responsible for the age-related reduction in osteoblast number (D’Ippolito et al., 1999). In addition, it has been demonstrated that cells cultured from human trabecular bone are not only osteogenic, but undergo adipocytic differentiation under defined culture conditions (Nuttall et al., 1998). A better understanding of the pathways triggered by Hh proteins is necessary to elucidate the mechanisms by which these proteins modulate...
osteofogenesis/adipogenesis in vitro and in vivo. The elucidation of these mechanisms could be crucial to consider new approaches to treat osteopenic disorders.

REFERENCES


