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# Inhibition of Histone Acetylation as a Tool in Bone Tissue Engineering

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## ABSTRACT

**Our approach to bone tissue engineering is the *in vitro* expansion and osteogenic differentiation of bone marrow–derived human mesenchymal stem cells (hMSCs) and their subsequent implantation on porous ceramic materials. Current osteogenic differentiation protocols use dexamethasone to initiate the osteogenic process, thus ignoring the multiple signaling pathways that control osteogenesis *in vivo*. Supporting osteogenesis at multiple stages might further enhance the bone-forming capacity of hMSCs. As reported previously, inhibition of so-called histone deacetylases (HDACs) stimulates osteoblast maturation, and in this report, we investigated whether trichostatin A (TSA), a widely used HDAC inhibitor, can be implemented in bone tissue engineering. We confirmed that TSA treatment of hMSCs results in increased expression of alkaline phosphatase (ALP) with concomitant increase in mineralization. Flow cytometry demonstrated that TSA increases the percentage of ALP-positive hMSCs as well as their average ALP expression level, but the robustness of the response differs between donors. Unfortunately, TSA has a profound negative effect on cell proliferation, so we investigated whether hMSCs respond to TSA after reaching confluence. Confluent hMSCs on tissue culture plastic displayed enhanced ALP expression. Therefore, we seeded TSA-treated hMSCs onto ceramic particles and analyzed ectopic bone formation upon implantation in immune-deficient mice. Unfortunately, TSA-treated hMSCs did not display better bone formation *in vivo* than control cells. Finally, we observed that TSA treatment strongly enhanced bone formation of *ex vivo* cultured mouse calvaria, which warrants further exploration of TSA in bone tissue engineering.**

## INTRODUCTION

HUMAN MESENCHYMAL STEM CELLS (hMSCs) are pluripotent cells from the bone marrow that can be expanded *in vitro* and differentiated into the osteogenic, chondrogenic, and adipogenic lineages[1] and into skeletal muscle cells.[2] MSCs were initially identified as the fibroblastic adherent fraction of bone marrow aspirates[3,4] and are also referred to as colony forming unit-fibroblasts (CFU-Fs), marrow stromal cells, bone marrow mesenchymal cells, and mesenchymal progenitor cells. In the presence of dexamethasone and β-glycerol phosphate, hMSCs express osteogenic markers such as alkaline phosphatase (ALP), and they deposit an extracellular matrix, which becomes mineralized under appropriate culture conditions.[5–9] Because of their ready availability and well-established *in vitro* culturing protocols, hMSCs have been the source of cells in autologous bone and cartilage tissue engineering.[10–13] For bone tissue engineering, we and others have demonstrated ectopic bone formation by seeding hMSCs onto porous calcium phosphate scaffolds and subsequently subcutaneously implanting them into immune-deficient mice.[14,15] We

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recently tested this therapeutic strategy in a Phase I clinical trial to treat patients with jaw defects. Although the procedure is safe, and bone formation was observed, the amount of bone formed needs to be enhanced to fully reconstruct the lost bone tissue (unpublished data). To further improve the biological activity of hMSCs, we look for molecular cues that can stimulate hMSC proliferation and differentiation *in vitro* and *in vivo*. Previously, we reported that stimulation of the Wnt signal transduction pathway can be used as a tool to enhance proliferation of hMSCs,[16,17] and we recently discovered that activation of the protein kinase A signaling pathway substantially enhances early osteogenesis with concomitant stimulation of bone formation (unpublished data). A number of reagents are available to stimulate matrix deposition and mineralization. The positive effect of ascorbic acid on collagen synthesis has been reported (see reference 18 and citations therein); and vitamin D3 stimulates the expression of the extracellular matrix component osteocalcin.[19] To further enhance this process, we investigated the effect of a group of chemical compounds known to interfere in the process of gene transcription. The chemicals inhibit a class of enzymes referred to as histone deacetylases (HDACs), which regulate histone to deoxyribonucleic acid (DNA) interaction.[20,21] In the absence of acetyl groups, histones bind to chromosomal DNA, thereby condensing it and thus making it transcriptionally less active. Upon covalent addition of acetyl groups using histone acetyl transferases (HATs), the histone–DNA interaction weakens, and the DNA becomes available for the transcription machinery. In contrast, HDAC activity enhances DNA–histone interaction, thus leading to gene silencing. HDACs are recruited to promoters and act as co-repressors of gene activity. In skeletal biology, HDACs have been involved as negative regulators of chondrocyte hypertrophy[22] by binding and inhibiting the activity of runt-related transcription factor 2 (runx2). Furthermore, binding of HDAC3 to runx2 leads to decreased osteocalcin gene expression and inhibition of osteogenesis.[23] In agreement with this, two recent papers describe that HDAC inhibitors stimulate the mineralization phase of mouse MC3T3 and hMSCs.[24,25] In this article, we investigate whether HDAC inhibitors can be employed as a tool in bone tissue engineering. We used trichostatin A (TSA) and valproic acid (VPA) because they are widely used as HDAC inhibitors for *in vitro* studies and both inhibitors have a mild effect when used *in vivo*. Valproic acid is a registered drug prescribed as an anticonvulsant and to treat bipolar disorders,[26] and TSA is considered to be a drug that may be effective in treating breast tumors.[27] We confirmed that TSA and VPA stimulate ALP expression by hMSCs and that TSA enhances mineralization. Possibly because of the negative effect of TSA on hMSC proliferation, TSA-treated hMSCs did not display enhanced bone formation upon implantation in immune-deficient mice. In contrast, TSA treatment strongly enhanced bone formation in *ex vivo* cultured mouse calvaria.

## MATERIALS AND METHODS

### *Cell culturing*

Bone marrow aspirates (5–15 mL) were obtained from donors who had given written informed consent. hMSCs were isolated and proliferated. Briefly, aspirates were plated at a density of $5\times10^5$ nucleated cells per $cm^2$ and cultured in hMSC proliferation medium, which consists of minimal essential medium (α-MEM, Life Technologies, Breda, the Netherlands), 10% heat-inactivated fetal bovine serum (FBS, Cambrex, Verviers, Belgium), 0.2 mM ascorbic acid (Asap, Life Technologies), L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 10 μg/mL streptomycin (Life Technologies), and 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, the Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% carbon dioxide ($CO_2$). Medium was refreshed twice a week, and cells were used for further subculturing or cryopreservation upon reaching near confluence. hMSC basic medium was composed of hMSC proliferative medium without bFGF, and hMSC osteogenic medium was composed of hMSC basic medium supplemented with $10^{-8}$ M dexamethasone (Sigma, Zwijndrecht, the Netherlands) and 0.01 M β-glycerol phosphate (Sigma). Goat MSCs were isolated from 30-mL bone marrow aspirates of Dutch milk goats.

Cell counting was performed manually using a Burker-Turk counting chamber or enzymatically using an Alamar blue assay. For the latter, culture medium was replaced with medium containing 10% Alamar blue solution (Bioscource, Camarillo, CA, USA), and cells were incubated at 37°C for 4 h. Fluorescence was measured in a 200-μl sample at 590 nm in an enzyme-linked immunosorbent assay plate reader (Greiner, Alphen a/d Rijn, the Netherlands).

### *Reagents*

A 400-mM lithium chloride (CalBiochem, San Diego, CA, USA) stock solution in α-MEM was used. Retinoic acid (Sigma) was dissolved in dimethyl sulfoxide (DMSO) as a 100× solution. Control cells were treated with 1% DMSO. TSA (Sigma) was kept as a 2.5-mM stock in DMSO and further diluted to a 20-μM work solution in phosphate-buffered saline (PBS). Valproic acid (Sigma) was dissolved directly in the appropriate medium. A 10-μg/mL bone morphogenic protein 2 (BMP2) solution (R&D systems, Abingdon, Oxon, United Kingdom) was prepared in 4 mM hydrochloric acid (HCl) and 0.1% bovine serum albumin (BSA, Sigma).

### *ALP flow cytometry and biochemical analysis*

The effect of HDAC inhibitors on ALP expression was studied using flow cytometry on cells seeded at 1000 cells/$cm^2$ in 6-well plates and grown under various conditions for 6 days. Each experiment was performed in triplicate and

included a negative control (cells grown in basic medium), a positive control (cells grown in osteogenic medium), and one or more experimental conditions. After 6 days of culture, cells were trypsinized and incubated for 30 min in PBS/5% BSA and incubated in PBS/1% BSA plus primary antibody (anti-ALP B4–78; Developmental Studies Hybridoma Bank, University of Iowa) or isotype control (DAKO, Heverlee, Belgium) for 30 min, washed 3 times in PBS/1% BSA and incubated with secondary antibody (goat anti-mouse immunoglobulin G phycoerythrin-conjugated, DAKO) for 30 min. Cells were washed 3 times and suspended in 250 μL PBS/1% BSA plus 10 μL Viaprobe (PharMingen, BD Biosciences, Alphen a/d Rijn, the Netherlands) for live/dead cell staining. Staining was analyzed using a FACS Calibur (Becton Dickinson Immunocytometry Systems, Alphen a/d Rijn, the Netherlands), and ALP levels were analyzed on living cells only. Percentage of ALP-positive cells and their ALP level was expressed relative to the isotype control.

Alternatively, ALP activity was detected using an enzymatic assay by incubation of the cells in 96-well plates for 15 min in a solution containing 20 mM para-nitrophenol phosphate (Sigma), 1 M diethanolamine (Sigma), 1 mM magnesium chloride plus 5.26 mg/mL Sigma $104^R$ phosphatase substrate (Sigma) at pH 9.8. Optical density was measured on a KC4 spectrometer. ALP activity was normalized to cell numbers, as determined by Alamar blue assay.

### *Mineralization*

For mineralization, hMSCs were seeded in duplo at 1000 cells/cm$^2$ in T25 culture flasks and grown under various conditions. In every experiment, osteogenic medium was used as a positive control and basic medium as a negative control. Mineralization after 28 days of culture was expressed as the amount of calcium deposited per T25. The total calcium deposition was assayed using a calcium assay kit (Sigma diagnostics; 587A) according to the manufacturer's protocol. Briefly, the culture medium was aspirated, washed twice with calcium and magnesium-free PBS (Life Technologies), and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected for direct measurement or stored at −20°C. The calcium content was measured at 575 nm (Perkin Elmer, Boston, MA, USA, Lamda 40) and expressed as μg of calcium/flask.

### *Quantitative polymerase chain reaction*

The effect of TSA on the expression of *ID1* and *ID2* was determined by seeding hMSCs of donors at 5000 cells/cm$^2$ in T25 culture flasks in 5 mL of proliferation medium and culturing until about 80% confluence. Then, medium was replaced by low-serum (0.5%) basic medium, low-serum basic medium supplemented with 20 nM TSA, and low-serum basic medium supplemented with 20 ng/mL BMP2 (R&D Systems) for 24 h. All conditions were in triplicate. Total ribonucleic acid (RNA) was isolated using an RNeasy mini kit (Qiagen, Venlo, the Netherlands) and on-column DNase treated with 10U Rnase-free Dnase I (Gibco, Paisely, United Kingdom) at 37°C for 30 min. DNase was inactivated at 72°C for 15 min. The quality and quantity of RNA was analyzed using gel electrophoresis and spectrophotometry. Two μg of each Dnase-treated RNA sample was used for first-strand complementary DNA (cDNA) synthesis using Superscript II (Invitrogen, Paisely, United Kingdom) according to the manufacturers' protocol. One μL of 100× diluted cDNA was used for 18S ribosomal RNA (rRNA) control amplification, and 1 μL of undiluted cDNA was used for *ID1* and *ID2* amplification. Polymerase chain reaction (PCR) reactions were performed and monitored on a Light Cycler real-time PCR machine (Roche, Woerden, the Netherlands) using the SYBR Green I master mix (Invitrogen Life Sciences, Paisely, United Kingdom) with primers for 18S rRNA (18srRNA-F 5′cggctaccaca tccaaggaa3′ and 18srRNA-R 5′gctggaattaccgcgggt3′), *ID1* F-5′gcaagacagcgagcggtgcg3′ and R-5′ggcgctgatctcgccgttga g3′, and *ID2* F-5′cctcccggtctcgccttcc3′ and R-5′ggttctgcccgg gtctctgg3′. PCR data was analyzed using Light Cycler software version 3.5.3, using the fit point method by setting the noise band to 1. Expression of genes was calculated relative to 18S rRNA levels using the comparative $\Delta C_T$ method.[28]

### *Ectopic bone formation*

As scaffold for bone tissue engineering, we used porous biphasic calcium phosphate (BCP) ceramic granules of approximately 2 to 3 mm, containing 20% β-tricalcium phosphate and 80% hydroxyapatite, which were sintered at 1200°C. Cells were grown in proliferation medium, and 3 days before the end of the culture, supplemented with 0, 5, or 20 nM TSA or 1 mM dibutyryl adenosine 3′-5′ cyclic monophosphate (cAMP, Sigma). Then, 9 granules were seeded with $2\times10^5$ cells of each group and implanted after 3 h. Before implantation, tissue-engineered samples were washed in PBS. Six immune-deficient mice (HsdCpb: NMRI-nu, Harlan, Horst, the Netherlands) were anesthetized using an intramuscular injection of a mixture containing atropine, xylazine, and ketamine. The surgical sites were cleaned with ethanol, subcutaneous pockets were created, and each mouse was implanted with 3 granules of each culture condition. At the end of the 6-week incubation period, the implants were removed and fixed in 0.14 M cacodylic acid buffer, pH 7.3, containing 1.5% glutaraldehyde. The fixed samples were dehydrated and embedded in methyl methacrylate, and sections were processed on a histological diamond saw (Leica SP1600, Wetzlar, Germany) and stained with 0.3% basic fuchsin solution; the samples were scored positive when red fuchsin stain was observed using light microscopy. The animals were housed at the Central Laboratory Animal Institute, Utrecht University,

and the Dutch Animal Care and Use Committee approved the experiments.

### *Mouse calvarial culture and micro-computed tomography analysis*

Calvaria were dissected from 8- to 10-day-old C56BL/6 mice (ICR-Harlan), and a 6-mm circle was punched out such that substantial sections of parietal bone and a small section of occipital bone were present. Calvaria were immersed in α-MEM supplemented with 10% heat-inactivated FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) and transferred to a 24-well plate containing 2 mL of medium (control), medium supplemented with 1 nM human recombinant interleukin-1β (IL1β, R&D Systems), with 1 nM IL1β plus $10^{-5}$ M TSA (Calbiochem) or 1 nM IL1β plus $10^{-7}$ M TSA. Plates were placed in an incubator at 37°C in a humid atmosphere with 5% $CO_2$. Calvaria were cultured for 13 days, and medium was changed 3 times a week. The bone volume was determined using micro-computed tomography (μCT) analysis using a Skyscan 1076 MicroCT-40 system (Skyscan, Aartselaar, Belgium). Skyscan software was used to collect the data. The X-ray tube was operated with photon energy of 70 kV, current of 140 μA, and a 0.50-mm-thick Al filter. Scanning width was 35 mm, and pixel size 9 μm. For scanning, calvaria were positioned as described[29] and imaged on the first day of the experiment, and bone volume was determined. Treatment groups were assigned based on individual calvaria volume; mean volume between groups was not significantly different ($n = 5$). Data are presented as percentage bone volume. Bone volume at the start of the experiment was set at 100%, and bone volume at day 13 was expressed as percentage compared with day 0. Mean values and standard deviations for control and experimental calvaria were compared using Graph Pad Prism. Statistical significance for the results was computed using one-way analysis of variance and Tukey's multiple comparison test. Differences were regarded as statistically significant for $p < 0.05$.

## RESULTS

### *TSA treatment of MSCs results in upregulation of ALP activity*

We approach bone tissue engineering using *in vitro* expansion of bone marrow–derived MSCs, followed by differentiation using the glucocorticoid dexamethasone. To investigate the potential use of TSA and VPA in our bone tissue engineering protocol, we first investigated its effect on hMSC osteogenesis. We treated hMSCs with increasing doses of the HDAC inhibitor TSA[30] and measured the expression of bone/liver/kidney-specific ALP using flow cytometry as an early osteogenic marker.[31] TSA was chosen because its activity has been well documented and because TSA shows mild toxicity *in vivo*.[32,33] As a positive control, we exposed hMSCs to dexamethasone, which resulted in a 3-fold increase in ALP level (Fig. 1A), demonstrating the osteogenic potential of our hMSC culture. Treatment of hMSCs with TSA also resulted in a dose-dependent increase in ALP levels. In the donor displayed in Fig. 1A, 20 nM TSA yielded an optimal expression level, whereas hMSCs from other donors displayed even higher expression at 50 nM (data not shown). When cells were co-treated with dexamethasone and TSA, we found an even stronger increase in ALP expression, up to 8.5-fold at 20 nM TSA. Similar results were obtained with cells from 4 donors (Fig. 5A). Because we frequently use the goat as a preclinical model to assess newly developed tissue-engineering approaches, we treated cultures of goat MSCs with TSA. Goat MSCs expressed higher levels of ALP upon treatment with TSA (Fig. 1B).

Next, we treated hMSCs with VPA.[26] We found that at 1 M, but not at 0.1 M, VPA was able to stimulate ALP expression (Fig. 1C). hMSCs grown at this high concentration of VPA displayed severe inhibition of proliferation (data not shown). Therefore, we decided to focus on TSA in our further studies.

### *The signaling context of TSA-induced ALP expression*

The positive effect of HDAC inhibitors on ALP expression by hMSCs has been reported previously.[24,25] Before focusing on the application of TSA in tissue engineering, we wanted to exclude that TSA-induced ALP expression is mediated through established osteogenic stimuli, such as BMP signaling or retinoic acid (RA) signaling.[34–37] First, we investigated whether TSA is able to initiate BMP signaling. We treated hMSCs with BMP2 or TSA for 24 h and analyzed expression of BMP target genes *ID1* and *ID2*. Whereas BMP2 treatment resulted in strong induction of both genes, TSA had no effect on them at all (Fig. 3A). Another signaling pathway modulated by HDAC activity is RA signaling.[38] Although RA is involved in osteogenic differentiation of mouse embryonic stem cells, the role of RA signaling in osteogenic differentiation of primary hMSCs is unknown. Therefore, we treated hMSCs with a concentration range of RA and analyzed ALP expression. We found that RA reduced ALP expression at all doses examined (Fig. 3B), confirming data by Ogston *et al.* using an immortalized MSC cell line.[39] Therefore, we exclude the possibility that TSA modulates ALP expression by enhancing RA signaling.

We previously reported that dexamethasone-induced ALP expression is sensitive to lithium-induced Wnt signaling.[16,17] To investigate whether TSA-induced ALP expression is lithium-sensitive as well, we co-treated cells with TSA and lithium for 7 days and analyzed ALP expression. As shown in Fig. 3C, TSA-induced ALP expression displayed lithium sensitivity similar to dexamethasone-induced ALP expression.

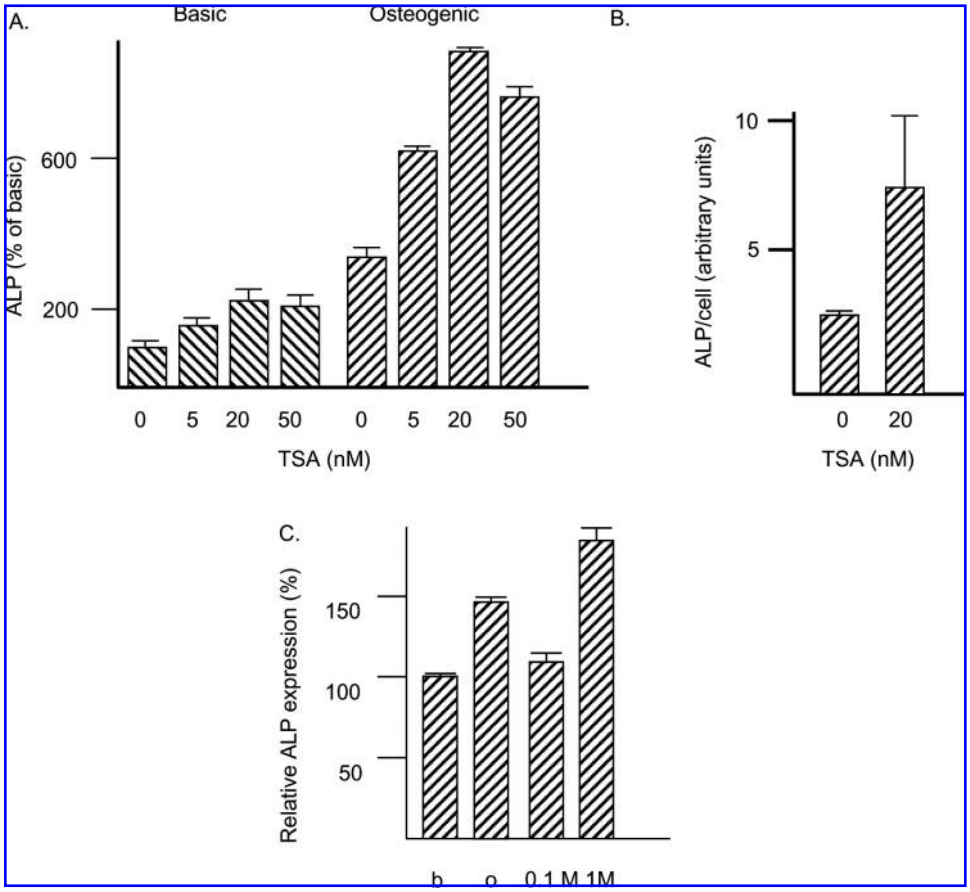


**FIG. 1.** Histone deacetylase inhibitors stimulate alkaline phosphatase (ALP) expression in human and goat mesenchymal stem cells (MSCs). (**A**) Dose-dependent increase in ALP expression assessed using flow cytometry on human MSCs grown for 6 days in the absence (basic) or presence of the osteogenic factor dexamethasone (osteogenic). Note greater ALP expression in osteogenic medium than in basic medium, which trichostatin A (TSA) further enhances. The experiment was repeated with cells of 4 other donors with similar results. Statistical significance relative to the control condition was determined using Student t-test ($p < 0.05$). (**B**) Increased ALP expression of goat MSCs cultured for 6 days in the presence of 20 nM TSA. ALP expression was measured in triplicate using a biochemical assay. Statistical significance relative to the control was determined using Student t-test ($p < 0.05$). (**C**) Cells grown for 6 days in the presence of 1M (1M) valproic acid show greater ALP expression than cells grown in basic medium (b) or osteogenic medium (o). All experiments were performed in triplicate, and standard deviation is represented by error bars. Statistical significance relative to the control condition was determined using Student t-test ($p < 0.05$) for the osteogenic and 1M valproic acid group.

### *TSA treatment stimulates mineralization but inhibits proliferation*

To further study the effect of TSA treatment on osteogenesis, we exposed hMSCs continuously to 20 nM TSA for 28 days and analyzed mineralization. Quantification of calcium deposition in cells from 2 donors revealed that dexamethasone strongly enhances mineralization (Fig. 2A), but treatment of hMSCs with TSA alone failed to induce mineralization. However, co-treatment of hMSCs with TSA and dexamethasone resulted in a mild or strong increase in mineralization, depending on the donor. Microscopic analysis of the mineralized plates at day 28 revealed that the TSA-treated culture flasks contained far fewer cells than those not exposed to TSA. We examined the effect of TSA on hMSC proliferation of various donors by exposing them to a range of TSA concentrations. Fig. 2B demonstrates that TSA dose-dependently inhibits proliferation. We found quantitative differences in the response of hMSCs of different donors, varying from mild to very strong responses to TSA, although we always observed the same trend (data not shown).

### *Pre-implantation treatment of hMSCs for tissue engineering purposes*

ALP expression plays a critical role in the mineralization process,[40] and we hypothesized that the positive effect of TSA on ALP expression and mineralization could be

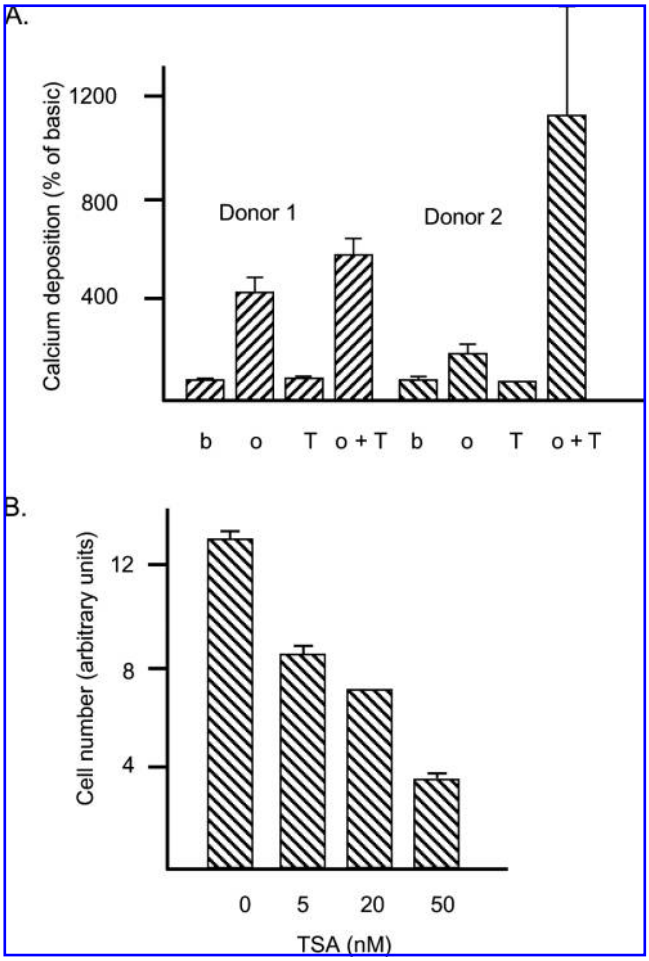


**FIG. 2.** Trichostatin A (TSA) stimulates dexamethasone-induced mineralization but inhibits proliferation. (**A**) Mineralization of human mesenchymal stem cells (hMSCs) after 28 days of culture in basic medium (b), osteogenic medium (o), basic medium with 20 nM TSA (T), and osteogenic medium with 20 nM TSA (o + T). Mineralization was quantified in triplicate using calcium assay in cells from 2 donors. Statistical significance of the o + T condition relative to the osteogenic condition was determined using Student t-test ($p < 0.05$). (**B**) Proliferation of hMSCs in the presence of several concentrations of TSA for 6 days. The experiment was repeated with cells from 3 other donors with similar outcome. All experiments were performed in triplicate, and standard deviation is represented by error bars. Statistical significance for all conditions relative to the control condition was determined using Student t-test ($p < 0.05$).

exploited to improve bone tissue engineering. Our current strategy is to isolate hMSCs from the bone marrow of donors and to expand them *in vitro*.[41,17] During expansion, we expose the cells to dexamethasone to initiate osteogenic differentiation. Next, we seed cells onto porous ceramic particles and then culture for a further 7 days in the presence of dexamethasone before implantation or, alternatively, implant them after 4 h of seeding on the materials.

Because TSA has a significant effect on hMSC proliferation, we tried to limit the time necessary to expose cells to TSA. Therefore, cells of 4 different donors were grown for 7 days in basic or osteogenic medium, and TSA was added in the last 3 and 6 days. Three days of exposure was sufficient to increase ALP expression in cells of all 4 donors (Fig. 4A), although the expression was higher after 6 days. An alternative strategy is to grow the cells until confluence and then expose them to TSA for several days. In Fig. 4B, we show that hMSCs grown to post-confluence display a robust response to dexamethasone treatment and that TSA has an additive effect to this.

### *Ectopic bone formation by TSA-treated hMSCs*

Based on the experiments described in the previous paragraph, we investigated bone formation by TSA-treated hMSCs by expanding the cells up to 70% confluence; exposing them for 3 days to 5 nM and 20 nM TSA, respectively; seeding them onto porous ceramics; and implanting the construct subcutaneously in immune-deficient mice after 4 h. As a positive control, we exposed one group with cAMP, a compound for which we have recently proven its positive effect on bone formation by hMSCs using this protocol.[42] As a negative control, cells were grown in medium without supplements. Because of the stringent conditions (implantation 4 h after seeding), no bone was observed in the control group (Fig. 5A). In contrast, bone formation was observed in all 6 samples of the cAMP-treated group. Unfortunately, bone formation was not observed in any sample of hMSCs treated with 5 or 20 nM TSA.

### *TSA stimulates bone formation in explanted mouse calvaria*

The previous studies show that exposure of hMSC to TSA *in vitro* does not benefit bone formation *in vivo*. To investigate whether TSA has a positive effect on bone formation *in vivo*, we explanted mouse calvaria and cultured them for 13 days in the presence or absence of TSA. We had previously found that the total bone mass, measured using μCT analysis, does not change during this period (data not shown). Therefore, we used the bone mass on the first day of the experiment as control. Furthermore, bone formation *in vivo* is the net result of bone formation by osteoblasts and bone resorption by osteoclasts. Therefore, we treated the calvarial cultures with IL1β, which is a strong stimulator of osteoclastogenesis.[29] As a result, we observed a 50% reduction in bone volume upon IL1β treatment (Fig. 5B). TSA was able to inhibit IL1β-induced bone loss at $10^{-5}$ M and $10^{-7}$ M. Moreover, at $10^{-5}$ M TSA, we observed a significantly greater increase in bone mass than in the control group. These data demonstrate that TSA does not only inhibit IL1β-induced bone loss but also stimulates bone formation in a calvarial culture model.

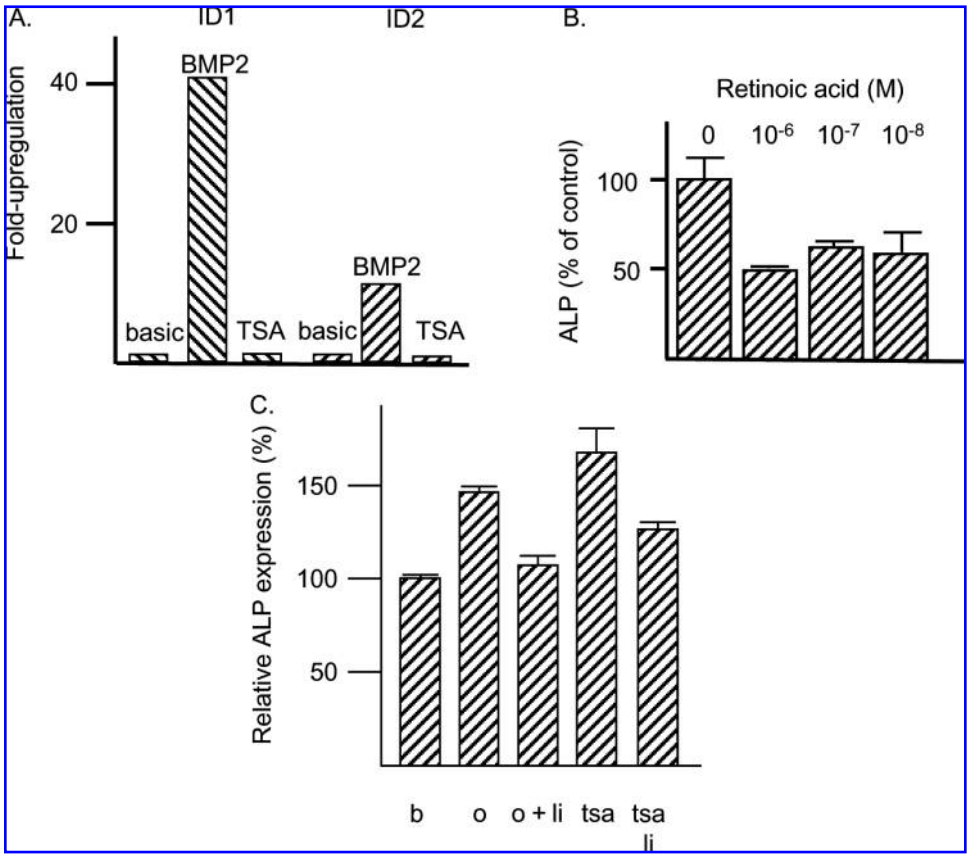


**FIG. 3.** Molecular context of trichostatin A (TSA)-induced alkaline phosphatase (ALP) expression. (**A**) Gene expression profile of bone morphogenic protein (BMP) target genes *ID1* and *ID2* in cells grown in basic medium (basic) or in basic medium supplemented with 20 ng/mL BMP2 or 20 nM TSA for 24 h. Experiments were performed in triplicate, error bars were too small to represent standard deviations. BMP2 did, but TSA did not, result in a statistically significant upregulation of *ID1* and *ID2* (Student t-test, $p < 0.05$). (**B**) ALP expression assessed using flow cytometry in human mesenchymal stem cells grown in triplicate in basic medium supplemented with 0, $10^{-6}$ M, $10^{-7}$ M, or $10^{-8}$ M retinoic acid for 7 days. Expression in basic medium was set to 100%. Error bars represent standard deviations. Student t-test indicated significant reduction in ALP level ($p < 0.05$) for all conditions relative to control. (**C**) TSA-induced ALP expression is sensitive to lithium-induced enhanced Wnt signaling. Cells were grown in triplicate in basic medium (b), osteogenic medium (o), osteogenic medium supplemented with 4 mM lithium chloride (o + li), basic medium plus 20 nM TSA (TSA), and basic medium supplemented with 20 nM TSA and 4 mM lithium chloride (tsa li). Lithium significantly inhibited dexamethasone- and TSA-induced ALP expression ($p < 0.05$).

## DISCUSSION

### *HDAC inhibitors stimulate osteogenic differentiation of hMSCs*

Histone deacetylases are a class of proteins involved in the regulation of transcription initiation of many different genes. Genetical studies have implicated HDACs in cardiac hypertrophy, cardiovascular defects, and the expression of certain class II HDACs are potential prognosis indicators for tumor progress (see reference 21 and citations therein). Moreover, HDACs have been implicated in skeletal defects. HDAC4-deficient mice display premature ossification of developing bones due to ectopic and early onset chondrocyte hypertrophy.[22] Also, the activity of several key regulators of osteogenesis, most noticeable cbfa1/runx2, is regulated by HDAC activity.[23] Schroeder and Cho describe a direct positive effect of HDAC inhibitors on osteogenesis of MC3T3 and hMSCs, respectively.[24,25] Based on these data, we decided to investigate whether HDAC inhibitors could be employed in bone tissue engineering. Our lab has used hMSCs as cell source for autologous bone tissue engineering.[15,16] Manipulation of the proliferation and differentiation phase of hMSCs has previously demonstrated substantial effect on the bone forming capacity of hMSCs *in vivo*[11,43,44] so we started our experiments by treating hMSCs with different HDAC inhibitors. Indeed, both trichostatin A and VPA had a dose-dependent positive effect on ALP expression. In more than 10 donors investigated we found that TSA stimulates ALP expression, although the cells from different donors do show a quantitative difference. The difference we observe in responsiveness to TSA, however, is very comparable to the heterogeneity reported in proliferation rate, response to dexamethasone (see Fig. 4A)

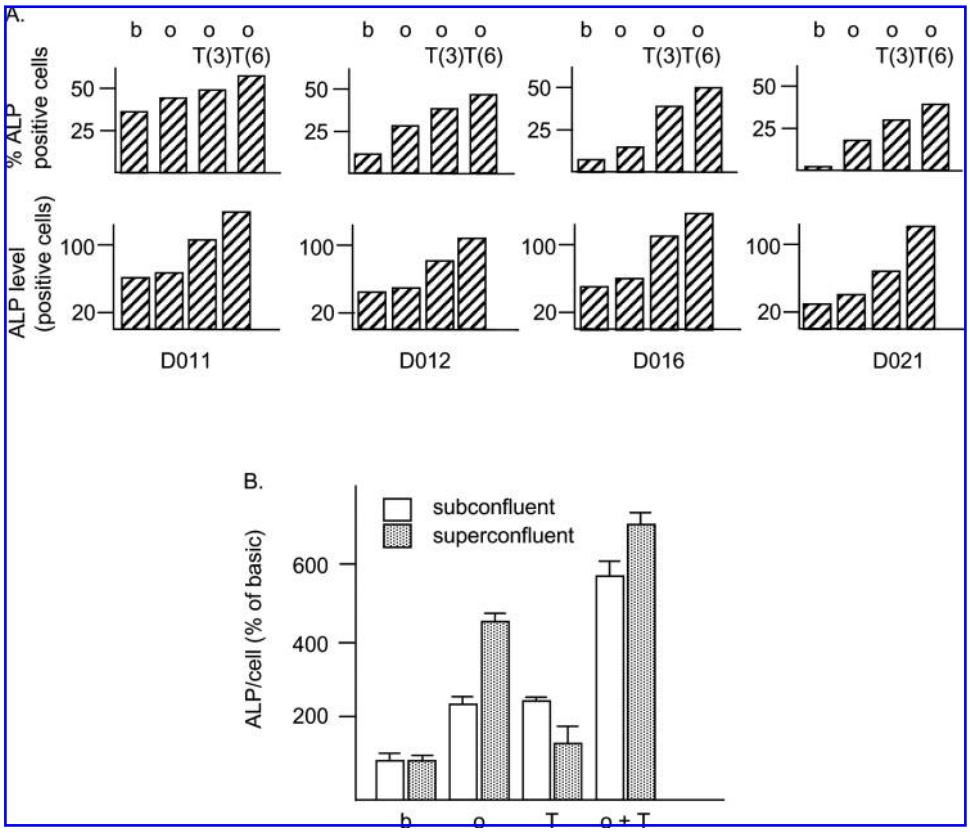


**FIG. 4.** Trichostatin A (TSA) treatment of human mesenchymal stem cells (hMSCs) before implantation. (**A**) Temporal expression of TSA is sufficient to stimulate the percentage of alkaline phosphatase (ALP)-positive cells, as well as the level of ALP in ALP-positive cells in hMSCs of 4 donors grown for 7 days in basic medium (b), 7 days in osteogenic medium (o), 4 days in osteogenic medium and then 3 days in osteogenic medium plus 20 nM TSA (o T3), or 1 day in osteogenic medium and 6 days in osteogenic medium plus 20 nM TSA (o T6). Note the inter-donor variation. The data represent the average of 2 experiments. (**B**) ALP expression was measured using biochemical assay in sub-confluent or super-confluent hMSCs grown in triplicate in basic medium (b), osteogenic medium (o), basic medium with 20 nM TSA (T), or osteogenic medium supplemented with 20 nM TSA (o + T). Error bars represent standard deviations. Student t-test demonstrated a significant effect of TSA on ALP expression in all conditions tested ($p < 0.05$).

or the potential to form bone *in vivo* by MSCs isolated from different donors.[43,44] In this respect, it is important to note that the combined administration of TSA and dexamethasone is able to reduce the difference between donors. As shown in Fig. 4A, hMSCs grown without osteogenic medium, the percentage of ALP positive cells varies between 2 and 39%, whereas cells treated with dexamethasone and TSA are 41 to 61% ALP positive. Besides increasing the percentage of positive cells, TSA also induced ALP level per cell. Interestingly, TSA alone was not able to induce mineralization, but we found a positive effect of TSA on dexamethasone-induced mineralization, which is similar to the effect of BMP2 on hMSC osteogenesis.[45] Cho *et al.* reported that the inhibitor of BMP, noggin, suppresses VPA-induced ALP expression in adipose tissue-derived MSCs,[25] which is surprising because BMPs do not stimulate ALP expression in hMSCs. To look into this, we investigated the effect of TSA on BMP target gene expression but found no induction of *ID1* and *ID2* after 24 h, whereas BMP treatment strongly induced them. We conclude that TSA does not directly stimulate BMP signaling, but we cannot exclude that TSA treatment results in upregulation of BMP gene expression and indirect activation of the BMP pathway. In conclusion, the signal transduction context of TSA-induced ALP expression remains elusive. Micro-array experiments with TSA-treated hMSCs could unveil the molecular details of TSA-induced osteogenesis.

### *Tissue engineering*

The result of TSA treatment on *in vitro* osteogenesis stimulated us to incorporate it into our bone tissue engineering protocol, which can be subdivided into 3 distinct phases: an expansion phase on tissue culture plastic, a differentiation phase on porous ceramic scaffolds *in vitro*, and a bone formation phase *in vivo*. We tried to delineate during which phase the cells should be exposed to TSA. We discovered that hMSC proliferation is sensitive to TSA; even doses as low as 5 nM lead to substantial inhibition of proliferation. In contrast,

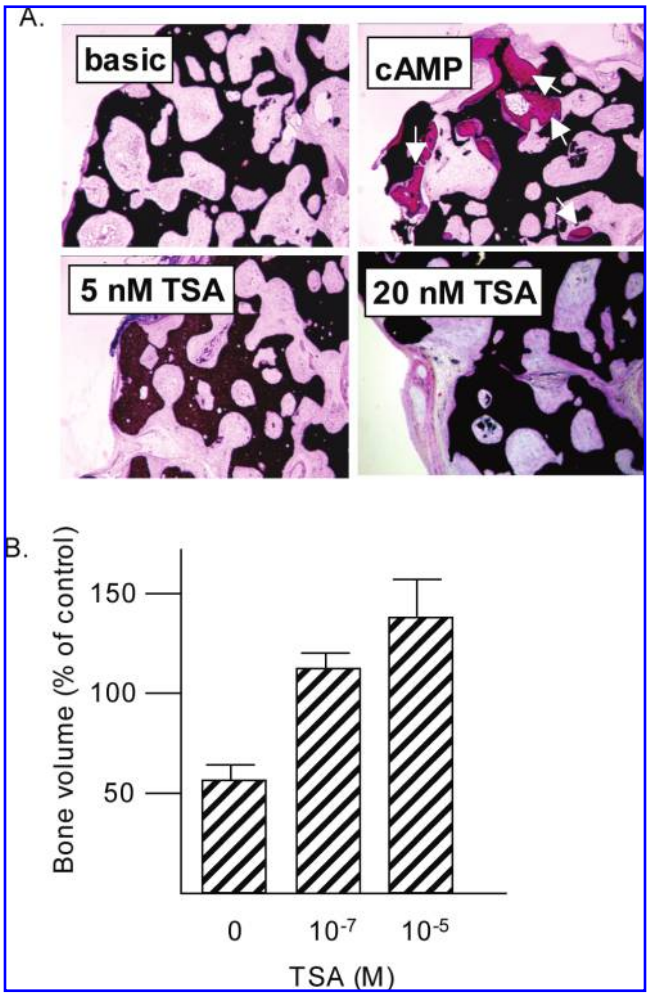


**FIG. 5.** Effect of trichostatin A (TSA) treatment on bone formation. (**A**) Three days TSA treatment before implantation does not stimulate ectopic bone formation by human mesenchymal stem cells (hMSCs). Alizarin red staining of histological samples of hMSCs treated with (TSA) or without (basic) TSA or with cAMP. Note extensive bone formation in cAMP-treated hMSCs, indicated by arrows. (**B**) Percentage calvarial bone volume (mean ± standard error of the mean) of mouse calvaria cultured with $10^{-9}$ M human recombinant interleukin-1β with or without 0, $10^{-7}$ M, or $10^{-5}$ M TSA ($n = 5$) relative to untreated control calvaria. Statistical analysis was performed using one-way analysis of variance followed by Tukey's Multiple Comparison Test and showed $p < 0.05$ for all groups. Color images available online at www.liebertpub.com/ten.

Schroeder *et al*. report that MC3T3 and primary mouse osteoblast proliferation is mildly sensitive to TSA, with 75% survival at 10 nM after 3 days.[24] Similarly, we found that mouse embryonic stem cell proliferation is relatively inert to TSA in the nM range (data not shown).

As such, cytotoxicity is a problem for tissue engineering because it will prolong the time that cells are in culture before implantation. As a compromise, we reduced the time that cells are exposed to TSA. Cells exposed for 3 days at the end of the expansion phase displayed enhanced ALP expression but not as high as those exposed for 6 days. When these cells were implanted in immune-deficient mice, we did not observe a positive effect on bone formation. We also tried to expose the cells to TSA during the differentiation phase. After seeding, cells on the scaffolds reach confluence and differentiate into the osteogenic lineage. Although post-confluent cells respond to dexamethasone, as indicated by enhanced ALP expression, TSA treatment did not result in further enhancement of ALP expression. Apparently, the cell cycle status is important to the sensitivity of hMSCs to TSA but not to dexamethasone-induced ALP. Therefore, we exclude the possibility of exposing hMSCs during the differentiation phase. The reason for the negative result on *in vivo* bone formation might be that the bone-forming assay was too stringent. We used a method in which cells were seeded onto ceramic particles and implanted 4 h later. Using this protocol, cells expanded in osteogenic medium fail to form bone (Fig. 5A and unpublished data), whereas cells expanded on the scaffolds for 7 days display bone formation.[44] However, cells expanded in the presence of cAMP formed bone (Fig. 5A and unpublished data), indicating that profound improvements in the protocol would have been detected. Another reason for the negative outcome could be that TSA negatively affects the bone-forming potential of hMSCs. We demonstrated that TSA stimulates ALP expression and mineralization *in vitro*, but we cannot exclude the possibility that TSA negatively affects the expression of genes that are critical for bone formation *in vivo*.

Finally, we tested whether TSA might have a positive effect on bone formation *in vivo* in a mouse calvarial explant culture system. Bone volume is the resultant of bone formation by osteoblasts and bone resorption by osteoclasts. We initiated bone resorption by the addition of IL1β, a stimulator of osteoclastogenesis. Addition of IL1β resulted in a 50% reduction of bone volume, but the addition of TSA could completely reverse this. Moreover, bone volume was even higher than observed in the control, clearly demonstrating the anabolic effect of TSA on bone formation. Two phenomena can explain enhanced bone growth in TSA-exposed calvaria. First, TSA may inhibit osteoclastogenesis. In line with this, Rahman *et al.* reported that osteoclasts are particularly sensitive to TSA.[46] Alternatively, our data, as well as the papers by Schroeder and Cho[24,25] support the hypothesis that TSA exerts its effect as anabolic agent by stimulating osteogenesis. We cannot exclude either possibility based on current data, and we have initiated a set of experiments to investigate how TSA skews the balance toward bone formation. In either case, TSA might be used as a stimulator of bone formation in bone-reconstruction procedures. Reports that underline increased fracture risk of epilepsy patients treated with VPA raise a point of concern.[47,48] The physiological reason behind this is not known but strongly discourages systemic exposure to HDAC inhibitors. Therefore, we are currently exploring local delivery of HDAC inhibitors to sites of active bone formation as an approach to exploit their anabolic activity.

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