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Purmorphamine and Oxysterols Accelerate and Promote Osteogenic Differentiation of Mesenchymal Stem Cells *In Vitro*

MICHAEL WÖLTJE[1*], MELANIE BÖBEL[2*], MAX HEILAND[3], BENEDICTA BECK-BROICHSITTER[3], AHMAD AL-DAM[3], ALEXANDER GRÖBE[3], REINHARD E. FRIEDRICH[3], HENNING HANKEN[3#] and RALF SMEETS[3#]

[1]*Institute of Textile Machinery and High Performance Material Technology, Dresden University of Technology, Dresden, Germany;*
[2]*Spintec Engineering GmbH, Aachen, Germany;*
[3]*Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

Abstract. *Aim: The purpose of the present study was to find inexpensive and non-toxic additives enhancing and accelerating the osteogenesis of mesenchymal stem cells in vitro, which can be used for tissue engineering of bone material. Materials and Methods: Osteogenic differentiation of rat mesenchymal stem cells was carried-out using classic differentiation medium containing or lacking purmorphamine, statins or oxysterols, respectively. Cell proliferation, alkaline phosphatase activity, calcium sedimentation and expression of bone matrix protein genes were measured to monitor differentiation. Results: Purmorphamine substantially suppressed proliferation, enhanced and accelerated alkaline phosphatase activity and calcium sedimentation and increased the expression of osteopontin and osteocalcin in rat mesenchymal stem cells in vitro. A similar osteogenesis-promoting effect was observed for oxysterols but not for the two statins. Conclusion: Purmorphamine and oxysterols promote and accelerate osteogenesis of mesenchymal stem cells in vitro suggesting their potential application for tissue engineering of bone material.*

The treatment of oral- maxillofacial bone defects is limited due to restricted therapeutic options. Currently, autologous bone graft represents the "gold standard" (1-3). Its decisive advantages are optimal immunological tolerance and the fact that regeneration of the transplant in the recipient site can be expected, especially when stimulated with growth factors. However, restricted availability, the risk of devitalization of the graft and damage of surrounding tissue, including a donor site morbidity, have to be considered as potential disadvantages of autologous bone grafts. Allogeneic bone material is available in greater extent and its application does not result in additional tissue damage at the donor site. Yet, since donor and recipient are genetically different, allogeneic transplants involve the risk of immunological reaction and transmission of infections. The requisite pre-treatment of allogeneic material with heat prior to transplantation leads to devitalization. Thus, the subsequent process of repair will inevitably result in a weakened dynamic structure of the implant due to resorption, combined with the necessity of a long discharge of the affected body part until sufficient stability is obtained.

Tissue engineering, and in particular with mesenchymal stem cells (MSC), recently opened a new perspective in obtaining autologous bone material and consequently offers a promising alternative to organ transplantation (4-7). The primary advantage of MSC is that they can be obtained without damaging the donor site, can be amplified *in vitro* to sufficient amount and then induced to differentiate into the desired tissue. For bone grafts, MSC of the bone marrow and periosteum are most suitable. After being expanded and induced to initiate osteogenic differentiation *in vitro*, pluripotent MSC are able to mimic the healing process of bone fractures (8-10). In combination with osteoblasts and a suitable matrix, MSC have been shown to initialize fracture-callus (8-10). In animal experiments, MSC cells have been successfully engrafted onto bone defect sites (11-15).

*These Authors contributed equally to this study.

#These Authors contributed equally to this study.

Correspondence to: Henning Hanken, MD, DMD, Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D–20246 Hamburg, Germany. Tel: +49 40741050006, Fax: +49 40741055467, e-mail: h.hanken@uke.de

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The standard method for inducing osteogenic differentiation of MSC is the combination of the glucorticoid dexamethasone with β-glycerophosphate and L-ascorbate (16). Several other growth factors, including bone morphogenetic proteins (BMP), are also known to induce osteogenic differentiation. However, recombinant growth factors are expensive and can be potentially toxic, especially at high doses (17). These limitations led to the development of other compounds, such as purmorphamine, a synthetic heterocyclic molecule, which has been shown to stimulate osteogenic differentiation in murine and human cells (18, 19). Statins, originally developed to lower cholesterol levels *via* inhibiting 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase, have also been shown to stimulate osteogenesis *in vivo* and *in vitro* (20-22). Another substance class with osteoinductive potential are hydroxycholesteroles. For example, oxysterols, products of cholesterol oxidation, promote and induce osteogenic differentiation in various cells, including osteoblasts (23). Combined application of oxysterols, 22(R)-hydroxy-cholesterole and 20(S)-hydroxycholesterole, elevated activity of alkaline phosphatase and induced mineralization *in vitro* (23, 24). However, all these substances are dependent on the presence of β-glycerophosphate and L-ascorbate in order to induce differentiation into mature osteoblasts, which are capable of forming a mineralized matrix.

The aim of this the present was to examine if addition of purmorphamine, statins or oxysterols to classic osteogenic differentiation medium could influence proliferation and osteogenic differentiation of adult MSC from rats *in vitro*.

Materials and Methods

Culturing and differentiation of mesenchymal stem cells (MSC). Isolation and *in vitro* cultivation of rat MSC were carried-out as previously described (25). Briefly, cells in bone marrow of Lewis rats were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose and 10 % fetal bovine serum (FBS) at 37˚C and 5% CO_2.

To induce classic osteogenic differentiation, standard medium was supplemented with 10 nM dexamethasone (Dex), 10 mM β-glycerophosphate (βGP) and 0.2 mM ascorbate-2-phosphate (AsaP). Purmorphamine (2 μM), the statines fluvastatin (0.5 μM), lovastatin (0.5 μM) and oxysterols 22(R)-hydroxycholesterole (5 μM) and 20(S) hydroxycholesterole (5 μM) were added to the specific media. All supplements were supplied by Sigma-Aldrich (Hamburg, Germany). Vital cell numbers were measured with CellTiter-Blue® reagent (Promega, Mannheim, Germany).

Evaluation of alkaline phosphatase (AP) activity. AP can be used as a marker of early-stage differentiation in osteoblasts. Determination of AP activity is based on an enzymatic reaction in which colorless p-nitrophenylphosphate (p-NPP) is converted to yellow p-nitrophenole. The resulting yellow dye can be measured photometrically at 405 nm. Absorbance is directly proportional to the activity of AP. To exclude serum AP activity, cells were kept in serum-free medium for 45 min before starting the assay.

Staining and quantification of calcium depositions using Alizarin red. Calcium deposition is the most important indicator for the differentiation of cells into osteoblasts. Sedimentations were quantified photometrically after staining of cell monolayers with 40 mM Alizarin Red S (Sigma-Aldrich) at pH 4.1 (26).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Gene expression of collagen type-I, osteopontin (*SPP1*) and osteocalcin (*BGLAP*) was examined using semiquantitative RT-PCR. RNA was isolated from cells in monolayer culture using the RNeasy-mini-kit (Qiagen, Hilden, Germany) and reverse-transcripted into cDNA using an Omniscript™ RT kit (Qiagen). RT-PCR was carried out at annealing temperature of 58˚C for 40 and 27 cycles for the collagen type-I and the osteocalcin cDNA, respectively. Glycerinaldehyde-3-phophate-dehydrogease (*GAPDH*) was used as control. Amplified fragments were separated on agarose gels. Primer sequences are available upon request.

Results

MSC were successfully isolated from bone marrow of three different donor Lewis rats. In the early stages of cell culture, heterogeneous cell clusters and elongated, spindle-shaped, as well as flat and bulky MSC were observed (Figure 1). MSC grew well in monolayer culture and confluent growth was reached within 9 days.

Cell proliferation. In contrast to continuous growth of cells in standard medium (Figure 2A), growth of cells in osteogenic differentiation-inducing media had two phases: an initial fast growing phase up to day 7 following a slower proliferation and even a stagnation of growth as observed for oxysterols. Similar growth dynamics were observed for cells grown in the presence of purmorphamine and oxysterols. Final cell numbers achieved with these two additives on day 21 were about 30% lower compared to classical induction. Cells grown in medium containing statins continued growth after day 12.

Activity of AP. In classic differentiation medium, AP activity increased by a factor of 2.5 until day 14 and then gradually decreased to its starting level (Figure 2B). Similar dynamics of AP activity were also observed in cells cultured in medium supplemented with purmorphamine (Figure 2D-F). Purmorphamine increased AP activity and accelerated the peak value from 14 to 10 days (Figure 2C). In contrast to purmorphamine, statines and oxysterols increased AP activity only by a factor of 1.5 to 2, respectively.

Calcium sedimentation. Calcium sedimentation was observed in classic differentiation medium on day 14 and increased to a maximum by day 21 (Figure 3B). In cells cultured with purmorphamine or oxysterols, calcium sediments could already be detected on day 7 (Figure 3C, F). On day 14, calcium sedimentations in the presence of additives were

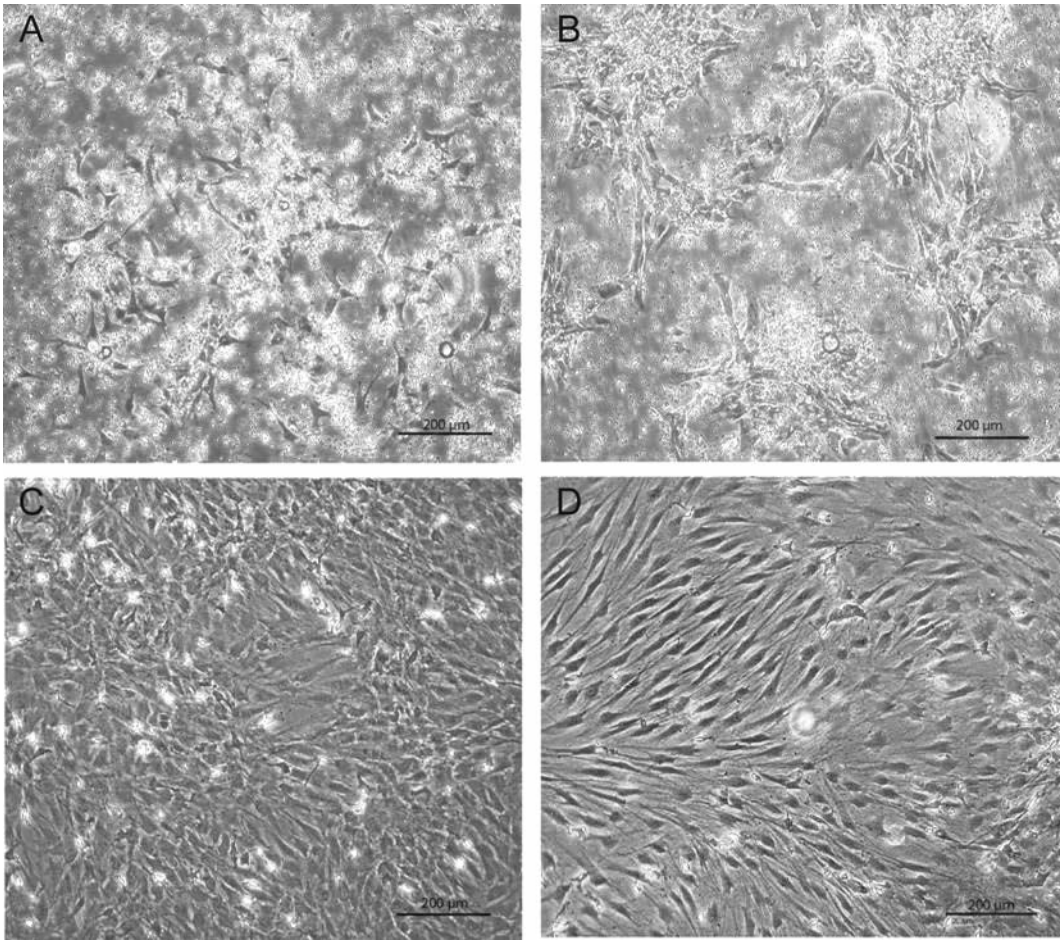

Figure 1. *Rat mesenchymal stem cells 4 days (A), 7 days (B) and 9 days (C, D) after isolation.*

higher than sedimentations in classic media achieved on day 21. Microscopic observation of the promoting and accelerating effect of purmorphamine and oxysterols on calcium sedimentation was confirmed by photometric measurement of the absorbance of the calcium-bound Alizarin Red S (Figure 4) revealing that addition of purmorphamine or oxysterols was able to nearly double calcium sedimentations compared to differentiation without. Lovastatin and Fluvastatin did not accelerate calcium sedimentation compared to classic differentiating medium (Figure 3E-F). No red calcium-nodules were visible under standard non-differentiating conditions (Figure 3A) over 21 days.

Expression of matrix protein genes. Since purmorphamine and oxysterols exhibited enhancing and accelerating effects in osteogenic differentiation of MSC, we further examined the expression of collagen type 1 gene, osteopontin gene and osteocalcin gene (Figure 5) only for these additives. Expression of collagen type I gene was observed in all cultures. The expression level on day 7 was substantially higher in the presence of purmorphamine or oxysterols. A shorter fragment of approximately 450-bp was found on day 21, possibly corresponding to a splice variant of the collagen type 1 mRNA. In every experimental group of differentiated cells, the osteopontin gene was expressed. The highest expression was detected on day 7 and continuously decreased afterwards. Addition of purmorphamine or oxysterols both increased the expression of the osteopontin gene. Purmorphamine and oxysterols induced osteocalcin gene expression already on day 7. In contrast, the gene expression of osteocalcin in the classic differentiation medium could not be detected before day 14.

Discussion

The results of our experiments showed an osteogenic differentiation-enhancing effect of purmorphamine like elevated AP activity, calcium sedimentation and expression of

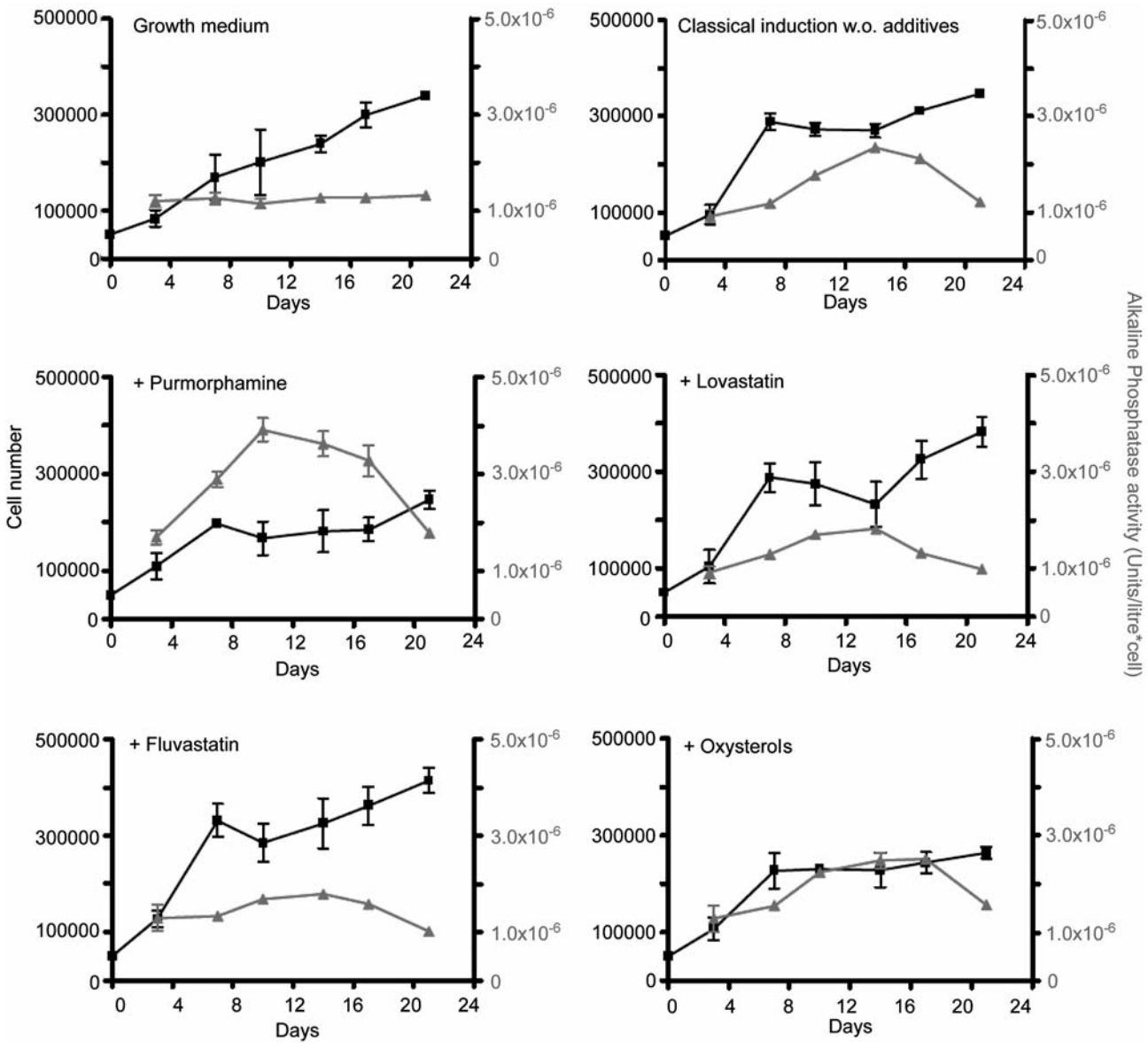

Figure 2. *Cell proliferation and activity of alkaline phosphatase of rat mesenchymal stem cells cultured over 21 days in standard growth medium and osteogenic-differentiation medium with or without various supplements, as indicated above the graphs.*

osteopontin and osteocalcin in MSC. In addition, oxysterols exhibited similar effects except enhanced AP activity.

During cell culture periods with media containing differentiating supplements, we observed a biphasic proliferation cycle with a plateau phase. This was in accordance to a report of Owen *et al*. (27). They postulated an observed decrease of cellular growth under differentiating conditions that corresponded to initiation of MSC differentiation and matrix protein synthesis. According to the differentiating model of MSC into osteoblasts, maturation leads to a proliferation arrest and initializes mineralization of the extracellular matrix (27). Our experiments were performed in classic differentiation medium supplemented with or without purmorphamine, statins or oxysterols. The presence of purmorphamine in cell culture medium increased AP activity on day 3 of the first proliferation phase. Nevertheless, the Alizarin Red S stain revealed only incipient mineralization in the beginning of the plateau phase. This early increase in AP activity was also seen in studies by Wu *et al*. and Beloti *et al*. (18, 19). However, in contrast to findings of Owen *et al*. (27), these two studies did not find a plateau phase during the differentiating cycle. Thus, stagnation of proliferation might

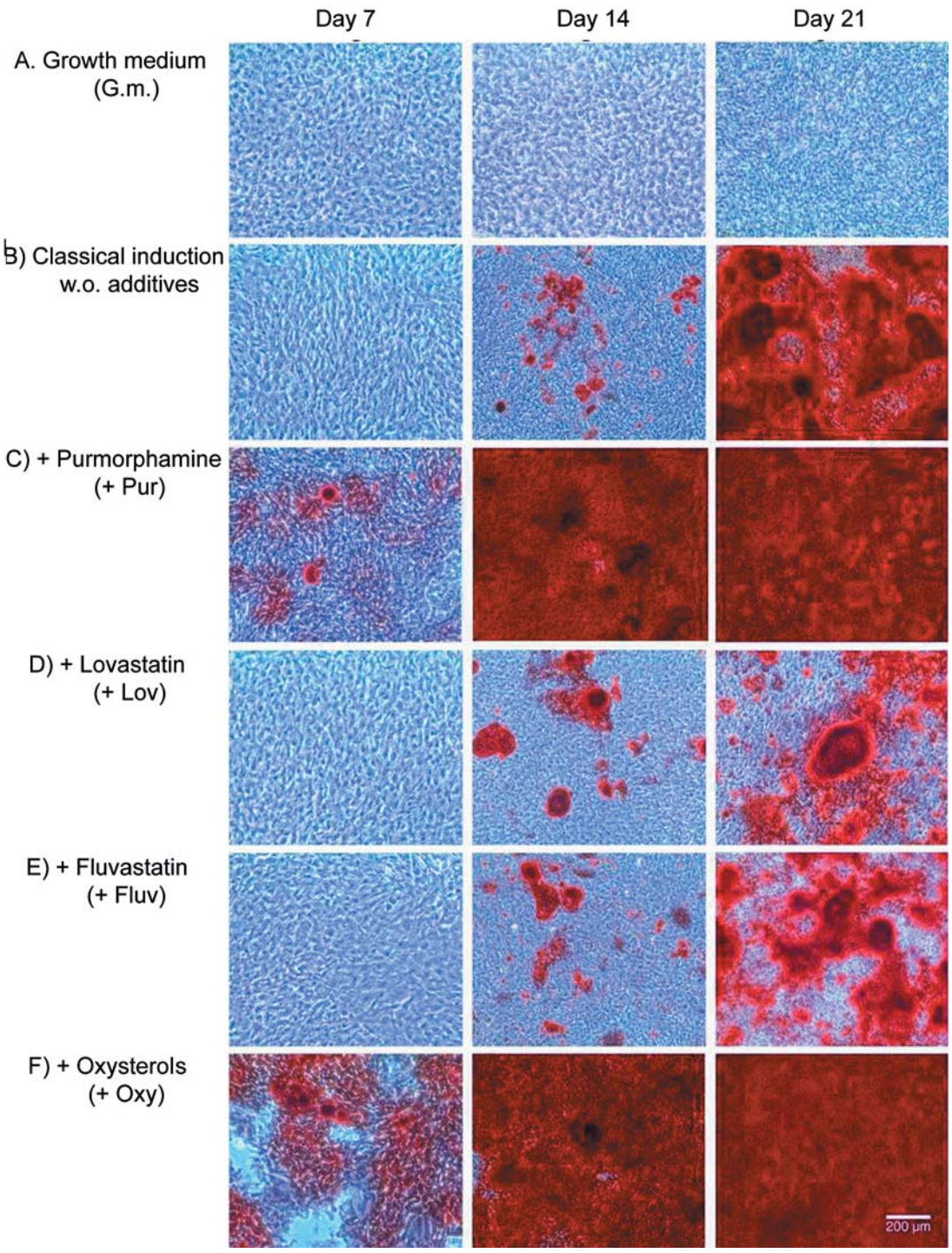

Figure 3. *Calcium sedimentation in cell monolayers by Alizarin Red S staining after 7, 14 and 21 days of culturing in various media.*

be an effect of dexamethasone, which was present in all cell culture media. This could be explained by activation of different signal pathways for osteogenic differentiation. Interestingly, cell proliferation was suppressed by purmorphamine or oxysterols suggesting an increased proportion of mature cells entering the differentiation phase.

Many studies showed that dexamethasone stimulates differentiation *in vitro,* as reviewed in Cheng *et al*. (28). In

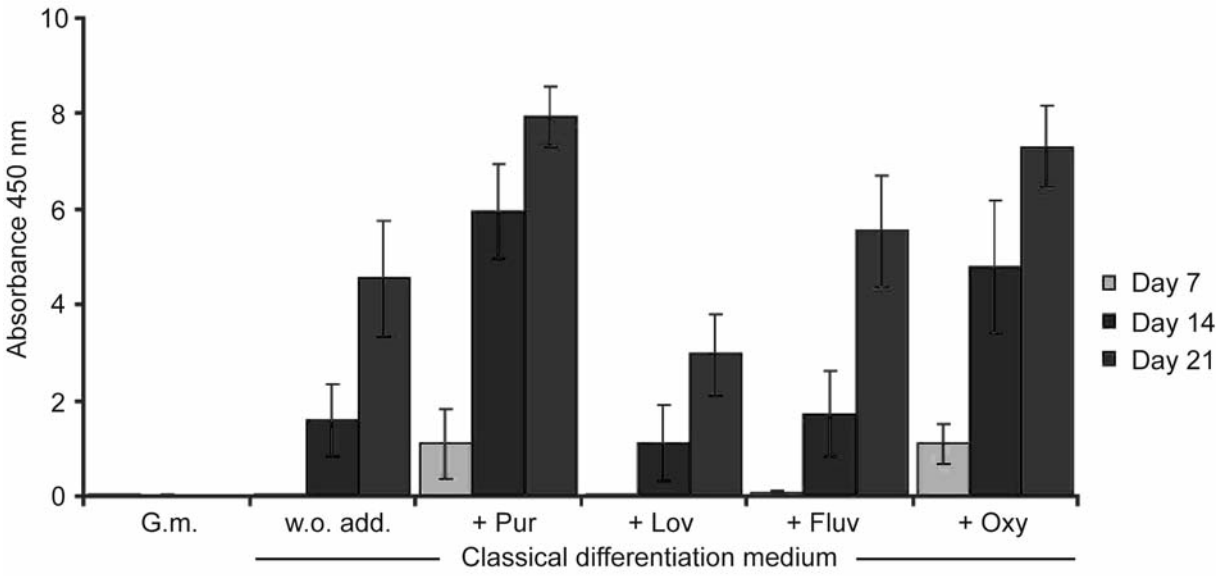

Figure 4. *Photometric quantification of calcium deposition stained by Alizarin-Red S after 7, 14 and 21 days of culturing mesenchymal cells in standard medium (G.m.), classical osteogenic differentiation medium without additives (w.o. add.) and supplemented with purmorphamine (+Pur), fluvastatin (Fluv), lovastatin (+Lov) or oxysterols (+Oxy). Values are means+standard deviation of 3 replicate cultures.*

contrast, high concentrations of glucocorticoids and prolonged incubation with dexamethasone lead to osteoporosis *in vivo* (29, 30). Furthermore, glucocorticoids inhibited the synthesis of osteoblastic molecules (31) and induced apoptosis of osteoblasts (32). Recent studies showed that dexamethasone inhibits the Wnt-signaling pathway (29). In addition, purmorphamine is known to activate the sonic hedgehog signal cascade and, thus, induces ostoblastic differentiation (33, 34). Furthermore, the increase in osteocalcin gene expression monitored in our PCR experiments was in good accordance to Fagihi *et al*. who reported an increase in osteocalcin gene expression in MSC cultures 14 days post-induction (35). Crosstalk of those two pathways may explain the additive or synergistic effect of purmorphamine and dexamethasone in suppressing proliferation and inducing osteogenic differentiation of the MSCs in our study.

In the present work, we achieved differentiation of MSC into cells of the osteoblast lineage producing calcium deposits in all tested differentiating media. However, the analysis of AP activity and Alizarin Red S staining showed that purmorphamine or oxysterols induced differentiation faster than the statins lovastatin or fluvastatin. Additional mRNA expression analysis using RT-PCR revealed a gradual increase of collagen gene expression *in vitro* during cell culture period. The osteopontin gene was already expressed strongly on day 7 and osteocalcin gene expression was detectable after 2 weeks.

For both differentiation schemes (purmorphamine or oxysterols) mineralization was detectable as early as 1 week after the initiation of osteogenic differentiation of MSC *in vitro,* as shown by Alizarin Red S staining. Both supplements were able to increase gene expression of i) collagen type I within 1 week, ii) osteopontin and iii) the late osteogenesis marker osteocalcin.

The observed positive effect of oxysterols on osteogenic differentiation of MSC was consistent with the results of Kim *et al*. who demonstrated that oxysterols induce the expression of the Notch target genes *HES-1, HEY-1*, and *HEY-2* in MSCs through activation of Hedgehog signaling and *via* a pathway independent of canonical Notch signaling (36). They concluded that although the Notch signaling pathway appears to play an important role in osteogenic differentiation and bone formation, the specific mechanisms of its actions, in this regard, remain undefined.

An important finding in our study is the acceleration of osteogenic differentiation by purmorphamine and oxysterols. Mineralization started one week earlier than without these reagents. Reducing the time needed for preparation of osteogenic differentiated, MSC is a decisive advantage for clinical applications.

Summarized, purmorphamine and oxysterols are able to accelerate osteogenic differentiation and increase the amount of mineral deposition compared to classical osteogenic differentiation media. Especially, purmorphamine is a low-cost drug that induced high AP activity. Thus, addition of purmorphamine may be used for faster supply of high amounts of osteogenic differentiated MSCs *in vitro* for clinical use. Therefore, it is a promising supplement for induction of osteogenesis of mesenchymal stem cells to obtain autologous bone material.

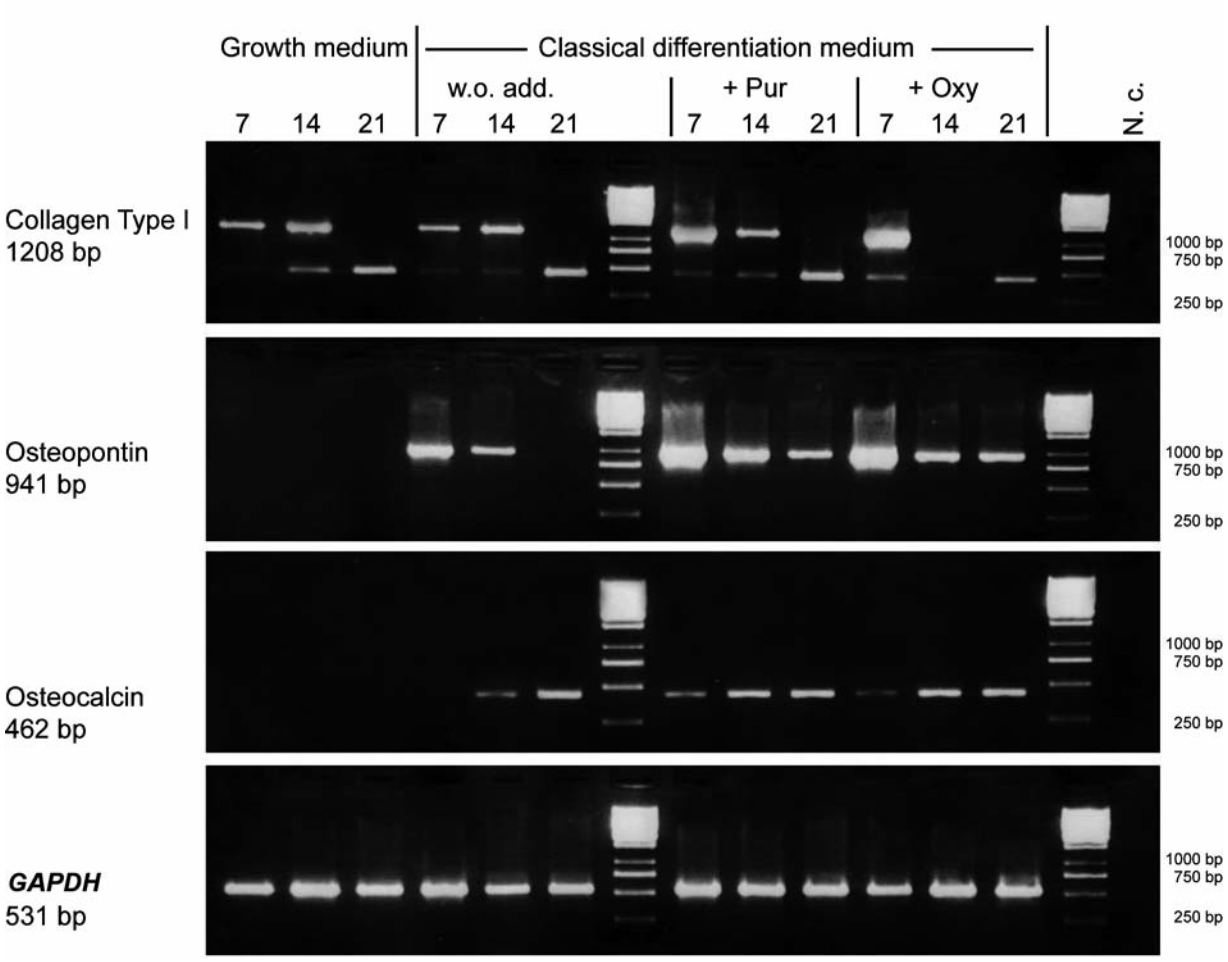

Figure 5. *Results of reverse transcription-PCR of relevant matrix proteins after 7, 14 and 21 days of cultures in standard growth medium and cultures in classical medium without additives (w.o.add), with purmorphamine (+Pur) and with oxysterols (+Oxy). GAPDH was used as a control. N.c. indicates no template control.*

Conflicts of Interest

The Authors declare that there is no conflict of interests regarding the publication of this paper.

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