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# Combinations of growth factors for human mesenchymal stem cell proliferation and osteogenic differentiation

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## Aims

**Here we introduce a wide and complex study comparing effects of growth factors used alone and in combinations on human mesenchymal stem cell (hMSC) proliferation and osteogenic differentiation. Certain ways of cell behaviour can be triggered by specific peptides – growth factors, influencing cell fate through surface cellular receptors.**

## Methods

**In our study transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) were used in order to induce osteogenesis and proliferation of hMSCs from bone marrow. These cells are naturally able to differentiate into various mesodermal cell lines. Effect of each factor itself is pretty well known. We designed experimental groups where two and more growth factors were combined. We supposed cumulative effect would appear when more growth factors with the same effect were combined. The cellular metabolism was evaluated using MTS assay and double-stranded DNA (dsDNA) amount using PicoGreen assay. Alkaline phosphatase (ALP) activity, as early osteogenesis marker, was observed. Phase contrast microscopy was used for cell morphology evaluation.**

## Results

**TGF-β and bFGF were shown to significantly enhance cell proliferation. VEGF and IGF-1 supported ALP activity. Light microscopy showed initial extracellular matrix mineralization after VEGF/IGF-1 supply.**

## Conclusion

**A combination of more than two growth factors did not support the cellular metabolism level and ALP activity even though the growth factor itself had a positive effect. This is probably caused by interplay of various messengers shared by more growth factor signalling cascades.**

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**Keywords:** Mesenchymal stem cell, Growth factor, Osteogenic differentiation, Regenerative medicine

## Article focus

- Does combination of more growth factors with a similar role lead to amplification of their effect? We used transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF).

## Key messages

- A combination of more than two growth factors showing positive effect did not itself lead to either metabolic activity or alkaline phosphatase (ALP) activity enhancement.

## Strengths and limitations

- Although the effects of certain growth factors are known, we used novel

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combinations (of up to three growth factors), the effects of which have not been published so far.
- In this study we have shown the significant positive effects of certain growth factor combinations; however, the whole phenomenon is probably much more complex and complicated, especially when discussing the in vivo conditions.

## Introduction

A general characteristic of mesenchymal stem cells (MSCs) is their ability of self-renewal and multilineage differentiation potential,[1] which indicates their main role in an organism-tissue structure and function restoration. MSCs are also of great potential for use in orthopaedics cell therapy once strict criteria are determined.[2] Differentiation of MSCs to a certain cell type can be induced by supplementation with specific growth factors, both of synthetic and natural origin, or other biomolecules.[3-5] Such substances influence cell metabolism, growth, proliferation, and differentiation by binding unique extracellular surface receptors.

Transforming growth factor β (TGF-β) expression is dependent on the amount of the collagens type I and II, both matrix proteins. It reduces production of osteoclast differentiation factor receptor activator of nuclear factor kappa-B ligand (RANKL) and thus controls the bone mass resorption.[6] Additionally, it activates osteoblast precursors and stimulates them to early differentiation and extracellular matrix (ECM) protein synthesis.[7] Basic fibroblast growth factor (bFGF) is synthesized by MSCs and mature osteoblasts and stored in the ECM. It regulates genes for osteoprogenitor proliferation and differentiation, and apoptosis of osteoblasts.[8] It also decreases noggin expression, which negatively regulates bone morphogenetic protein dependent bone formation.[9] Insulin-like growth factor 1 (IGF-1) was shown to influence mature bone cells supporting osterix, osteocalcin, and collagen type I expression. It also modulates chondrocyte functions and is crucial for proper endochondral ossification.[10] As an inactive molecule it is stored in bone ECM and released during bone resorption.[11] Vascular endothelial growth factor (VEGF) is especially important from the perspective of blood supply during bone development and healing. In the primary osteoblasts VEGF stimulates alkaline phosphatase (ALP) activity and mineralization. The maximal expression level is reached in the terminal phase of differentiation.[12] In the osteoclast precursor cells it induces higher receptor activator of nuclear factor kappa-B (RANK) expression, which functions as a RANKL receptor protein and enables precursor activation and activates osteoclast generation.[13] Hepatocyte growth factor (HGF) activates the signalling cascade leading to higher expression of RunX2, osterix, osteocalcin, and osteoprotegerin messenger RNA (mRNA), followed by phosphorylation. It also induces the expression of bone morphogenetic protein 4, another factor important for osteogenesis.[14,15]

In the field of tissue engineering the main aim is to mimic physiological conditions in order to facilitate the development of a new tissue, which would be a fully-fledged substitution of the originally present tissue. With this aim, some bioactive substances and growth factors in certain concentrations can be added to the culture medium. Common osteogenic differentiation protocol includes cultivation of the confluent cell layer with supplementation by synthetic glucocorticoid dexamethasone, β-glycerophosphate as an inorganic phosphate source, and ascorbate as a cofactor for lysine and proline hydroxylation.[16]

Bone formation is accompanied by ECM mineralization. This process is initiated by the secretion of tiny vesicles containing high concentrations of calcium and phosphates, and these are also the initial sites for hydroxyapatite crystal deposition.[17,18] Vesicles usually contain ALP.[19] ALP is in the outer membrane of the exocytosed vesicle anchored by the glycosylphosphatidylinositol. This enzyme catalyzes mineral deposition via decreasing extracellular pyrophosphate level and increasing phosphate level. The mineralization always starts at the ends of collagen fibres.

The aim of this study was to find the optimal combination of growth factors supporting human mesenchymal stem cell (hMSC) proliferation and osteogenic differentiation induction. It would be possible to use this combination for the functionalization of scaffolds mimicking the natural 3D cell microenvironment. We designed the groups with the aim to compare the effect of each growth factor itself and in combination with others in order to see if any process enhancement is present when more growth factors with the same effect are used together. We tried to stimulate cell proliferation and early differentiation by bFGF and TGF-β[7,8] and subsequently support the differentiation process by HGF, IGF-1, and VEGF, which are supposed to induce osteogenesis.[10,12,14] As far as we know a study describing comparison of such a growth factor combination range has not yet been published.

## Methods

**Cells and culture conditions.** We used commercially available hMSCs isolated from bone marrow (Human Bone Marrow-derived Mesenchymal Stem Cells, Catalog #7500; ScienCell, Carlsbad, California, USA). Cells were seeded in the third passage in 96-well culture plates at a density of 5,000 cells/cm$^2$. We performed five biological replicates. The cells were cultivated in Minimum Essential Medium (51,411 C; Merck/MilliporeSigma, St. Louis, Missouri, USA) with 10% Fetal Serum Albumin (F75224; Merck/MilliporeSigma) and 1% penicillin/streptomycin (15140 to 122; Invitrogen, Carlsbad, California, USA). Osteogenic medium was made by enrichment of basal medium with 10 mM β-glycerophosphate (50020; Merck/MilliporeSigma), 100 nM dexamethasone (D4902; Merck/MilliporeSigma), and 100 μM ascorbate-2-phosphate (A8960; Merck/MilliporeSigma). Growth

**Table I.** Composition of culture medium in the experimental groups.

| Number of group | Culture medium composition |
|---|---|
| 1 | negative control - osteogenic medium (OM) |
| 2 | OM+ HGF (20 ng/ml) |
| 3 | OM+ IGF-1 (10 ng/ml) |
| 4 | OM+ TGF-β (10 ng/ml) |
| 5 | OM+ bFGF (10 ng/ml) |
| 6 | OM+ VEGF (1 ng/ml) |
| 7 | OM+ TGF-β (10 ng/ml)+ bFGF (10 ng/ml) |
| 8 | OM+ VEGF (1 ng/ml)+ HGF (10 ng/ml) |
| 9 | OM+ VEGF (1 ng/ml)+ IGF-1 (10 ng/ml) |
| 10 | OM+ HGF (20 ng/ml)+ IGF-1 (10 ng/ml) |
| 11 | OM+ TGF-β (10 ng/ml)+ bFGF (10 ng/ml)+ VEGF (1 ng/ml) |
| 12 | OM+ TGF-β (10 ng/ml)+ bFGF (10 ng/ml)+ HGF (20 ng/ml) |
| 13 | OM+ TGF-β (10 ng/ml)+ bFGF (10 ng/ml)+ IGF-1 (10 ng/ml) |
| 14 | OM+ HGF (20 ng/ml)+ IGF-1 (10 ng/ml)+ VEGF (1 ng/ml) |

bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

factors were bought from PeproTech (London, UK) with the following catalogue numbers: bFGF–100 to 18 C, TGF-β - 100 to 21, HGF – 100 to 39, IGF-1 – 350 to 10, and VEGF – 100 to 20. These were added in the combinations and concentrations stated in Table I. Culture medium was changed every third day to maintain freshness. The same concentration of growth factors was used for the full duration of the experiments. Cells were cultivated for 21 days at 37 °C and 5% $CO_2$.

**Cell proliferation analysis by MTS test.** The MTS test is used to evaluate the level of cellular metabolism and viability. Yellow MTS substrate (G3581; Promega, Madison, Wisconsin, USA) – tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymehoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is incubated with the cells. It is reduced to insoluble purple formazan by cellular succinate dehydrogenase. To each well 20 µl of MTS substrate and 100 µl of culture medium was added. Samples were further incubated for one hour at 37 °C and 5% $CO_2$. Then an absorbance of 100 µl of product at 490 nm was spectrophotometrically measured, reference wavelength 690 nm. Cells cultivated in a culture medium without osteogenic supplements were used as a negative control.

**Alkaline phosphatase activity evaluation.** ALP activity was measured spectrophotometrically using a substrate for this enzyme (N7653; Merck/MilliporeSigma) p-nitrophenyl phosphate (pNPP). ALP catalyzes the conversion of a colourless substrate to the yellow product p-nitrophenol. Cells were washed twice with PBS and then 100 µl of ALP substrate was added. Samples were shielded from direct light and incubated for 15 minutes at room temperature. Following this, the whole volume of the solution was transferred to a new well and 50 µl of 2 M NaOH was added to stop the reaction. Finally, the absorbance of the product was measured at 405 nm. Cells cultivated in culture medium without osteogenic supplements were used as a negative control.

**Cell proliferation assay.** After incubation with the ALP substrate (N7653; Merck/MilliporeSigma) cells were washed twice with PBS and 200 µl of a lysis buffer was added. The lysis buffer consists of 10 mM Tris (T1503; Merck/MilliporeSigma), 1 mM ethylenediaminetetraacetic acid (EDTA) (EDS; Merck/MilliporeSigma), and 0.0004% Triton X-100 (T8787; Merck/MilliporeSigma). Cells were then frozen in wells. After thawing, cells were scrubbed from the bottom of the well by a pipette, transferred to a 1.5 ml tube, and vortexed. Samples were further frozen, thawed, and vortexed in two more cycles. Finally, 200 µl of working solution Quant-iT PicoGreenR dsDNA Assay Reagent (Q33120; Invitrogen) was transferred to a black 96-well plate with a transparent bottom, then 10 µl of sample, respectively DNA standard from the assay, was added. There is a fluorescently labelled probe in the working solution which starts to emit a signal after binding. The fluorescence was measured at excitation wavelength 485 nm and emission 528 nm.

**Light microscopy.** On the 21st day of the experiment samples were visualized using phase contrast microscopy on Olympus IX51, 100× magnification.

**Statistical analysis.** The obtained data were analyzed with one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test with the level of significance $p \leq 0.001$ (marked with the number of the group plus an asterisk (*)) and $p \leq 0.05$ (marked with the number of the group only). The number in the graph description marks in comparison with which group reached a certain group of significantly higher value. Use of "significant/significantly" in text always refers to statistical significance.

## Results

**Supplementation with TGF-β/bFGF supports cellular metabolic activity.** The cell metabolic activity in each experimental group was examined using MTS test on days 1, 10, 15, and 21 (Figure 1). As apparent, the additions of TGF-β or TGF-β combined with bFGF stimulated cells to increase metabolic activity. On the other hand, supplementation with VEGF, HGF, and IGF-1 led to lower metabolic activity than in the control group (no. 1), even on day 1. The metabolism level was growing continuously in groups containing the combination of TGF-β and bFGF regardless of which growth factor they were supplied with, except for IGF-1. In the experimental groups treated with HGF, IGF-1, and VEGF alone or in combination, the metabolic activity remained on approximately the same level during the whole experiment, and we also observed a decrease on days 15 and 21. Changes in metabolic activity are shown in Figure 1. In the graph there is a stated comparison of certain growth factor itself versus the same growth factor combined with others. We have presented the data in five different graphs in order to make the differences easier to see.

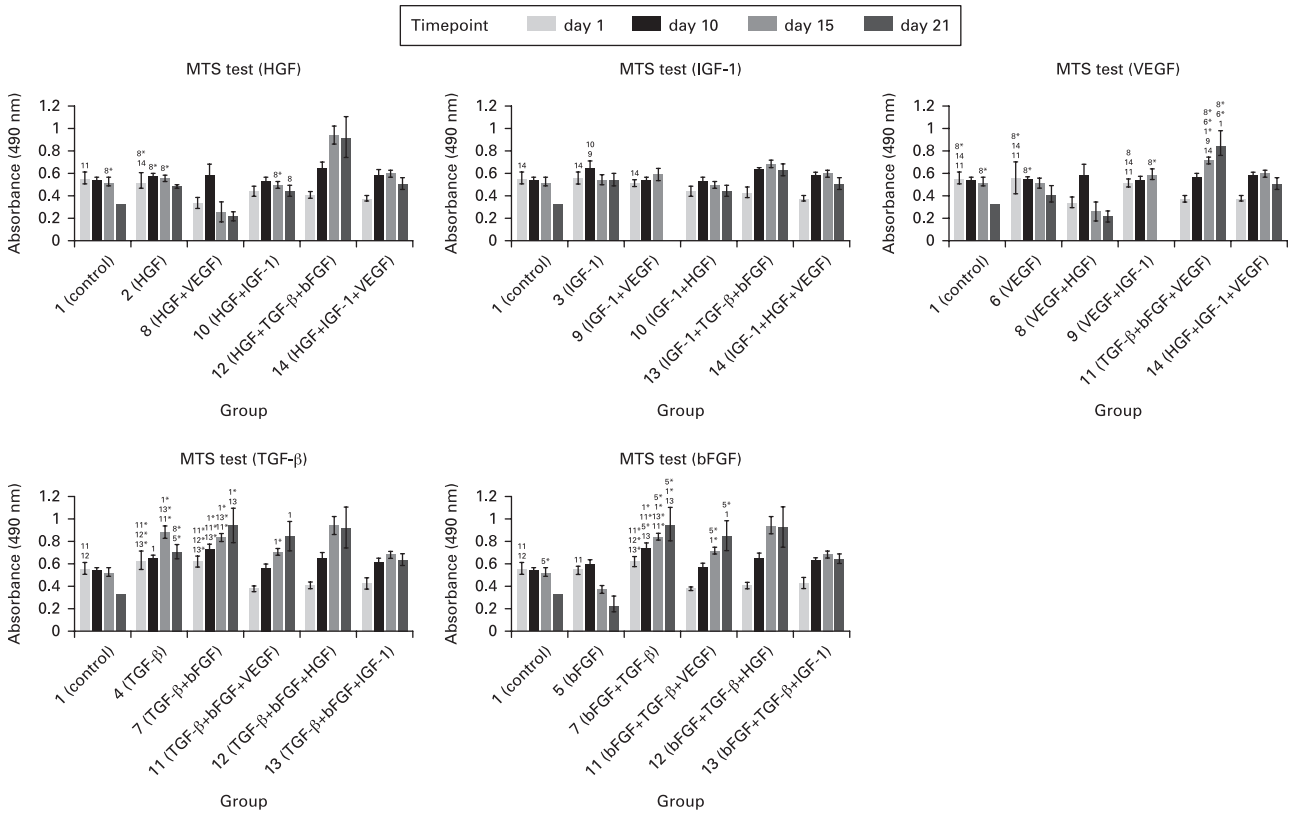


Fig. 1

Metabolic activity of cells evaluated by MTS test. Absorbance measured at 490 nm. Each graph shows a comparison of the growth factor effect itself (stated in the graph heading) and in combination with others (stated on the x-axis). Statistically significant differences are marked with the number of certain groups. Significant increase was observed in groups treated with transforming growth factor β (TGF-β) only or TGF-β with basic fibroblast growth factor (bFGF). $p < 0.05$ marked with the number of the group; $p < 0.001$ marked with the number of the group plus an asterisk (*). HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor.

**Double-stranded DNA (quantification).** For evaluation of the cellular proliferation level, we used PicoGreen assay with fluorescent probe. We obtained data indicating that in the groups 1, 2, 3, 4, 5, and 10, significantly more cells than in the remaining experimental groups were detected 24 hours after seeding (Figure 2). We did not observe a continuous double-stranded DNA (dsDNA) amount increase and despite the high SDs we observed significant differences. On day 10 there was a decrease of cellular dsDNA in all groups except group 8. A statistically significant difference was measured in group 3 in comparison with groups 7 and 12. Cells started to proliferate on day 15 in all groups. Significant differences were detected in groups 4, 6, 7, 8, and 13. The highest amount of DNA was present in groups 7, 8, and 13. There was no increase of cell proliferation shown on day 21. However, on the last experimental day we could see an increase of dsDNA in some samples in comparison with the first day. In the graph there is a stated comparison of a certain growth factor itself versus the same growth factor combined with others. We have presented the data in five different graphs in order to make the differences easier to see.

**Supplementation with TGF-β/bFGF decreased alkaline phosphatase activity while HGF/IGF-1 caused an increase.** The activity of ALP was measured in order to evaluate the level of stimulation of osteogenic differentiation by growth factors. In groups supplemented with the combination TGF-β/bFGF we measured significantly lower levels of ALP activity. In cases where these two growth factors were not used, the ALP activity rose steeply between days 1 and 10. As an early marker of osteogenic differentiation, ALP reached its maximal activity on day 10 in most of the experimental groups. When the cells were supplemented with TGF-β, activity of ALP was growing continuously during the whole experiment lasting for 21 days. This indicates that the whole process of osteogenic differentiation was delayed. Changes in ALP activity are shown in Figure 3. In the graph there is a stated comparison of a certain growth factor itself versus the same growth factor combined with others. We have presented the data in five different graphs in order to make the differences easier to see.

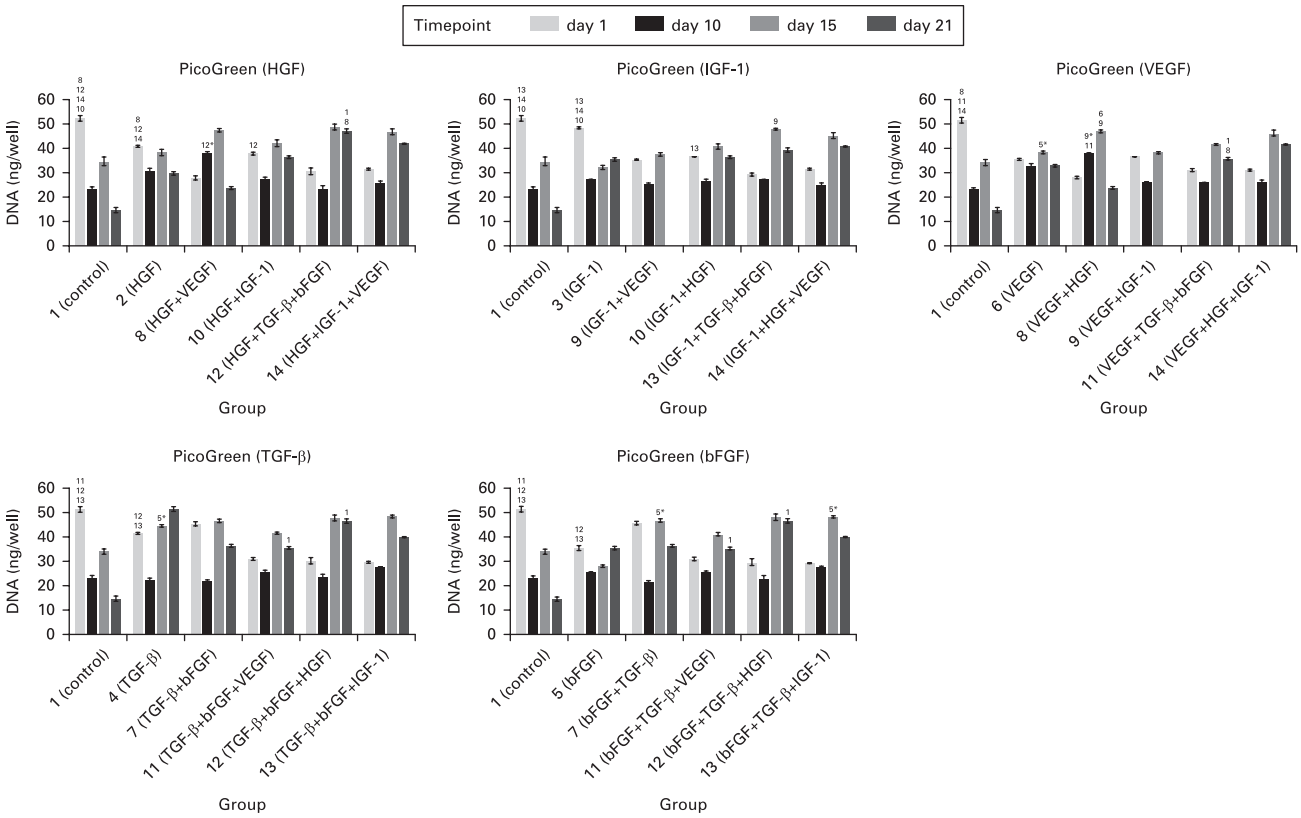


**Fig. 2**

Quantification of cellular double stranded DNA (dsDNA). Each graph shows a comparison of the growth factor effect itself (stated in the graph heading) and in combination with others (stated on the x-axis). As visible from the day 1 data, the initial adhesion was not equally successful in all groups. Therefore, the number of cells varied among groups. Some statistically significant differences were measured on day 15. In general, we obtained data with high SDs. Statistically significant differences are marked with the number of a certain group. $p < 0.05$ marked with the number of the group; $p < 0.001$ marked with the number of the group plus an asterisk (*). bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

On the last day of the experiment, we used a light microscope to visualize the cells and see their morphology (Figure 4). In groups where the cells were cultured in osteogenic medium alone or with additions of HGF, IGF-1, and VEGF, tiny 3D structures, equally on the whole surface of the cell culture, were visible. Cells in all groups were confluent by this experimental day.

## Discussion

The osteogenic differentiation of MSCs in vitro can be induced by the addition of growth factors and other bioactive substances to the culture medium. Common osteogenic medium with defined supplements was used for this purpose. In this study, the osteogenic medium was used as a basic culture medium with the aim to provide a sufficient inflow of mineral substances to the cells. Therefore, the cells can deposit mineral substances in their ECM or use them as a source of inorganic phosphate – ALP substrate, or ascorbate as a collagen synthesis cofactor. We assume that the use of the osteogenic medium approximated culture conditions to the in vivo situation in the manner of spectrum of biomolecules influencing cells composition. In this case MSCs would have been influenced by growth factors in the basal medium, ALP would be activated, but without follow-up calcium and other mineral substance accumulation or osteogenic marker expression.[20] In studies examining osteogenic differentiation of MSCs, growth factors were added to the culture medium separately or in combinations.[14,21-24] In our study we were investigating the effects of TGF-β, bFGF, VEGF, IGF-1, and HGF.

For some of the growth factors used, an ability to induce even other types of differentiation has been shown. Both TGF-β and bFGF were used for cartilage and tendon formation induction.[25,26] The cells showed morphological characteristics, specific tissue mRNA expression, and protein synthesis.[26,27] These are all connective tissues. They are especially functionally related, and besides that fact, both cartilage and tendons temporarily occur in spots of new bone formation. This could explain the heterogenous effect of particular growth factors on MSC differentiation. During the process of intramembranous ossification, a ligamentous tissue occurs. MSCs originating from the neural crest differentiate into osteoblasts and start to produce bone ECM components – collagen and proteoglycans which bind calcium salts. Osteoblasts

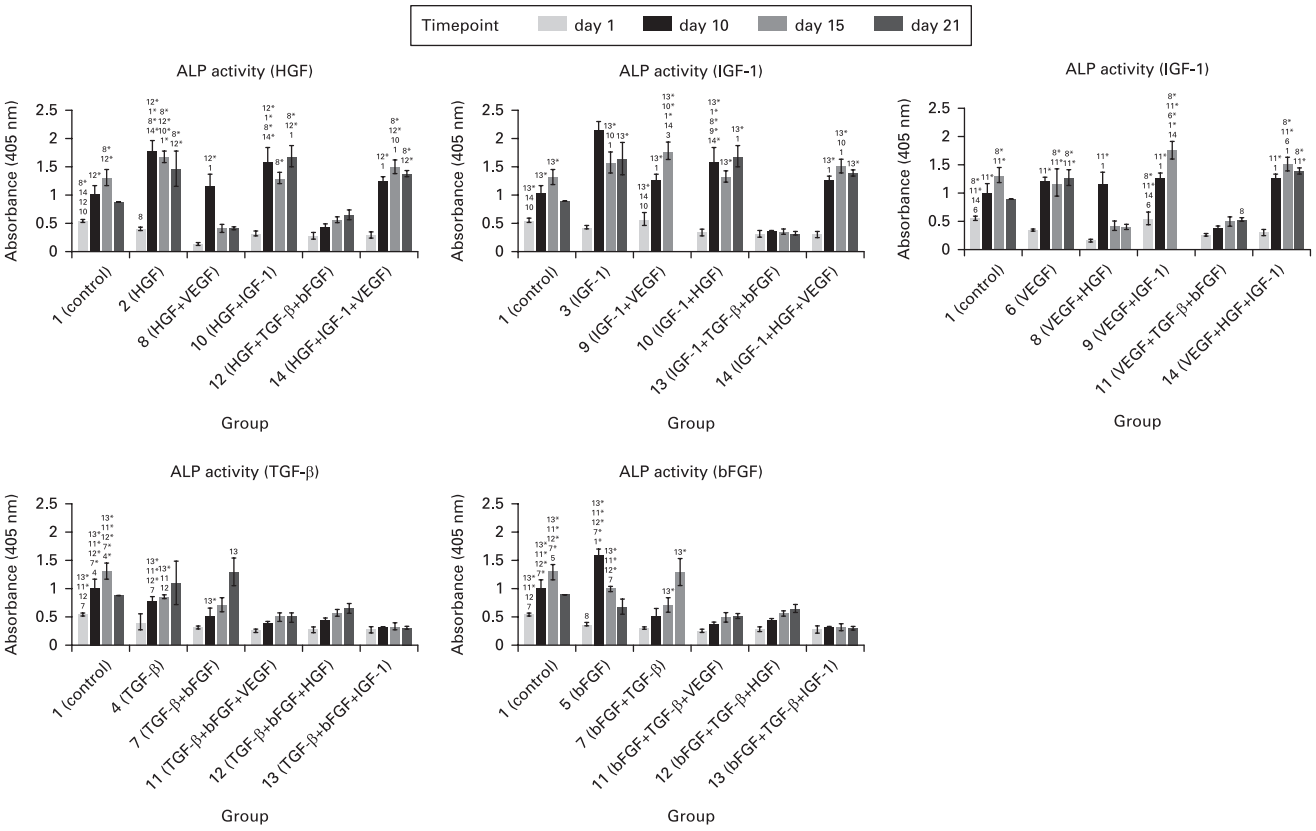


**Fig. 3**

Cellular alkaline phosphatase activity. Absorbance measured at 405 nm. Each graph shows comparison of the growth factor effect itself (stated in the graph heading) and in combination with others (stated on the x-axis). Significantly higher alkaline phosphatase (ALP) activity was observed in groups treated with hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) only, or in combination even with vascular endothelial growth factor (VEGF). Low ALP activity was observed in groups treated with basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β) used separately or in combination even with other growth factors. Statistically significant differences are marked with the number of a certain group. $p < 0.05$ marked with the number of the group; $p < 0.001$ marked with the number of the group plus an asterisk.

subsequently migrate to the calcified areas, so-called ossification centres, and are gradually closed in newly forming ECM. Entrapped cells transform into osteocytes. Finally, ligamentous periosteum is developed on the flat bone surface.[28] On the contrary, endochondral ossification involves hyaline cartilage, which is replaced by bone in the later phases. MSCs have activated transcription factors Pax1 and scleraxis leading to chondrogenic gene expression. Chondrocytes then condense through n-cadherins and Sox9 transcription is triggered. They consequently proliferate, synthetize cartilaginous matrix, and become hypertrophic. Fibronectin and collagen type X are synthesized, which allows calcium deposition. Simultaneously, the hypertrophic chondrocytes secrete vesicles containing enzymes generating calcium and phosphorus ions which initiate matrix mineralization in the primary ossification centres. In the final stages some chondrocytes die by apoptosis and others become osteoblasts and change their anaerobic metabolism to aerobic, following which the cartilage is replaced by bone.[29]

A positive effect on cellular metabolic activity measured by MTS test was observed for bFGF and TGF-β (Figure 1). If these two molecules are combined, they work synergistically and potentiate each other, which is the same conclusion as in studies dealing with their mutual interactions.[30] A common molecule in their signalling cascades is Ras GTPase, whose activation leads to proliferative gene transcription. Due to the fact that this GTPase is also part of signalling pathways of distinct receptor types, there are most likely some other molecules ensuring TGF-β and bFGF summation. As apparent from Figure 1, a more significant increase of cellular metabolism was observed after TGF-β addition. This growth factor supports cell viability and its positive effect on MSC proliferation has also been proven.[31] The principle of its action is the promotion of osteoprogenitor cell proliferation by extracellular signal-regulated kinase–mitogen-activated protein kinase (ERK-MAP-kinase) cascade stimulation.[32] This fact should manifest as an increase of the amount of cellular DNA detectable by PicoGreen. According to some other studies, TGF-β has an opposite effect – inducing senescence of the cells by increasing reactive oxygen species production in mitochondria.[33] The biomolecule bFGF is also supposed to have a mitogenic effect.[34] This claim was not validated with any significant differences, yet the graphs documenting cellular metabolic activity

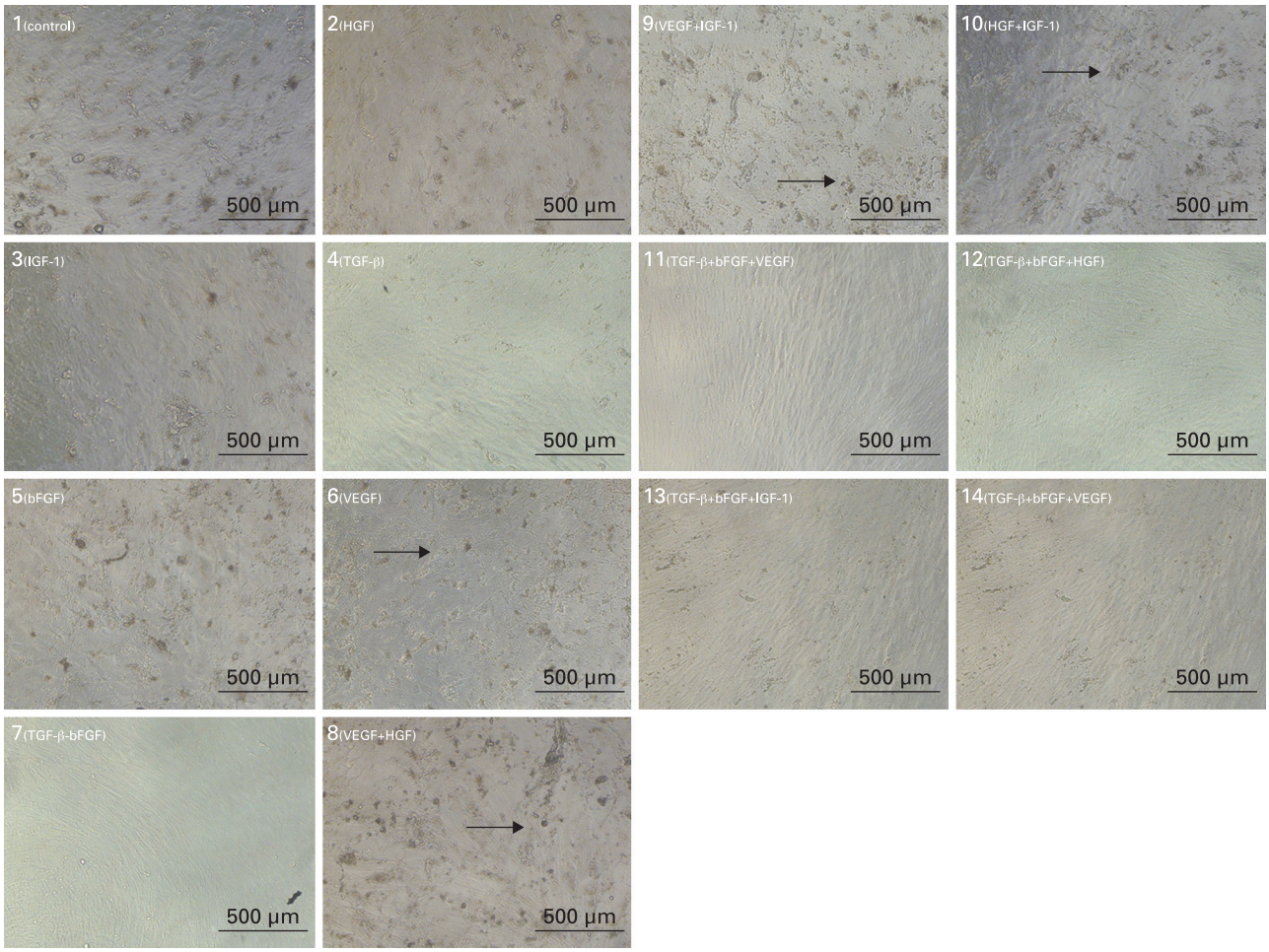


Fig. 4

Photographs from light microscope, 21st experimental day. Scale bar 500 µm. Photos showing initial phases of extracellular matrix (ECM) mineralization in groups treated with hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) alone or in combination even with other growth factors. Arrows point to the mineralization sites. bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

development show this trend in certain experimental groups. Synergistic function of TGF-β and bFGF was so strong that even the significant decrease of metabolic activity caused by the addition of some negatively acting growth factors was not big enough to decrease the effect of certain combinations to the level of the control group.

Cell metabolism was significantly decreased by combination of VEGF and HGF. If added separately, the same level of cellular metabolism as in the control group was measured. The data for VEGF obtained in this study by MTS test were in conflict with other published studies, where the positive effect on metabolic activity and proliferation of the cells was shown.[35,36] On the contrary, HGF is supposed to inhibit MSC proliferation.[37] In this case MSCs were treated by both growth factors, in some part of the cascades there was the most likely growth factors cooperation and effect modulation. The point of contact and signal amplification described by Sulpice et al[38] is in synergistic activation of the same set of ERK1/2 and p38 kinases. The resulting decrease of cellular metabolism can be a consequence of the higher number of activated molecules or of different kinetics of kinase stimulation.

A decrease of the amount of dsDNA measured in some groups in the last days of cultivation could be caused by the complexity of insufficient surroundings. As cells were cultivated on plastics, they were not sufficiently stimulated by physical interactions of the 3D microenvironment, which would significantly improve cell viability. In addition, using a 3D microcarrier system can possess advantageous gas diffusion properties.[39]

ALP activity, as an early osteogenesis marker, should grow until day 14 of cultivation. During the experiments we measured significantly higher ALP activity in groups with VEGF, IGF-1, and HGF in comparison with the untreated group. Stronger stimulation was observed for IGF-1 and VEGF. These data match with already published results.[22,40-42] On the other hand studies showing an opposite effect have also been published.[21,41,43] A decrease of ALP activity was observed in groups treated with combination TGF-β and bFGF, which is in agreement with results

of other studies dealing with MSC osteogenic differentiation.[21,30] ALP activity is stimulated by Smad3, a TGF-β signalling cascade molecule. Thus, for ALP activity reduction another cascade that does not include Smad3 has to be used. Sowa et al[44] showed that ALP activity can also be regulated by cascades through JNK and ERK1/2 kinases. As apparent from Figure 2, which compares cultivation with and without certain growth factors, an effect of the same molecule was not always the same. It depended on the remaining growth factors added in the culture medium. A combination of IGF-1, which supported ALP activity by itself, with TGF-β or bFGF caused a statistically significant decrease in comparison with TGF-β/bFGF alone. Also, the combinations HGF+ VEGF and HGF+ IGF-1 did not have the expected effect. We predicted these two to strongly support ALP activity due to the common signalling molecules in their cascades, however lower values were measured. When the combination of HGF+ IGF-1+ VEGF was used, the values were comparable with HGF in combination with IGF-1 alone.

After ALP activity increases, ECM mineralization then occurs. ALP hydrolyzes pyrophosphate and inorganic phosphate, which forms hydroxyapatite with the production of calcium ions. Hydroxyapatite crystals are deposited in the collagen fibre net from approximately the 14th day of cultivation.[29] Microscopic structures are visible on the photographs from the light microscope on day 21. These appear as grains among and on cells, whereas cells in the remaining experimental groups seem to be smooth. These structures are present in the same experimental groups where higher ALP activity was measured. Therefore, we assume them to be early signs of ECM mineralization. The cells were also present in a confluent layer, which is a crucial step foregoing mineralization.

In conclusion, our study showed a positive effect of HGF/IGF-1 on ALP activity and a positive effect of bFGF/TGF-β on metabolic activity of hMSCs. However, it is necessary to note that the entire study was done using hMSCs from bone marrow only. Thus, a possible limitation of the study is an MSC-specific reaction to the growth factors. There is always certain variability in cell behaviour associated with the cell type that needs to be considered.

The field of tissue engineering is evolving rapidly. In five years, we will probably be working with non-cellular carriers enriched with specific biomolecules influencing cell fate. The bioactive molecules will stimulate patients' own cells to proliferate and heal the defect completely with structurally and functionally restored tissue. This will enable fast and safe treatment even of complicated defects. The materials used will be especially synthetic in order to prevent disease transfer and immune system reaction.

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