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The Effect of Aging and Culture Senescence on Fibroblast Proliferation and Osteogenic Differentiation

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Abstract

Objective: The use of autologous cortical and cancellous bone remains the gold standard of bone-grafting. However, donor side morbidity, limited availability, and the risk of infections lead surgeons and researchers to seek suitable alternatives. Human fibroblasts are potent immunoregulatory cells with multipotent differentiation potential, which are easy to harvest and proliferate *in vitro*, which makes them attractive tools for bone tissue engineering. But for an autologous application in cell-based therapies, attention should be paid to the effect of donor age on differentiation potential. Culture senescence must also be considered, as some proliferation steps are necessary to obtain a sufficient cell number for therapeutic use.

Methods: The results of this study reveal that an additional supplementation of insulin-like growth factor 1 is more suitable for osteogenic differentiation of foreskin fibroblasts, evaluated with an alkaline phosphatase assay, and quantification of calcium deposition in the extracellular matrix.

Results: Our findings demonstrate that increasing donor age and culture senescence negatively affect the proliferation and osteogenic differentiation capacity of foreskin fibroblasts. These results suggest that the best approach to increase cell numbers is to optimize the seeding density, while additional growth factor application has no beneficial effect on the proliferation in early passages, analysed with a cell viability assay.

Furthermore, commonly used osteogenic differentiation strategies consist of an application of ascorbate-2-phosphate, dexamethasone, and β-glycerophosphate. However, phenotypic and differentiation potential discrepancies exist between multipotent mesenchymal stromal cells from different tissue origins as well as among fibroblasts from different dermal origins.

Conclusion: This work illustrates, that human fibroblasts, provided by young donors and in early cell culture passages, are a viable cell source for bone tissue engineering.

Keywords: Aging; Culture senescence; Fibroblast; Osteogenesis; Proliferation

Abbreviations: MSC: Mesenchymal stromal cells; FB: Fibroblasts; IGF 1: Insulin-like growth factor 1; RNA: Ribonucleic acid; P: Passage; CD: Cluster of differentiation; FBS: Fetal bovine serum; IL: Interleukin; TNF-α Tumor necrosis factor-α; IFN-γ: Interferon-γ; FGF-2: Fibroblast growth factor-2; TGF-β: Transforming growth factor-β; Deta-NO: Diethylenetriamine-nitric oxide; OM: Osteogenic differentiation media; ALP: Alkaline phosphatase; PCT: Polymerase chain reaction; 5′Aza: 5′-Azacytidine; BMP-6: Bone morphogenic protein-6; DNA: Deoxyribonucleic acid; Dexa: Dexamethasone.

Introduction

Bone tissue engineering is a promising research field in regenerative medicine, as bone is one of the most frequently transplanted tissues [1]. Classically, bone tissue engineering strategies consist of a combination of osteoconductive scaffolds and cells with osteogenic differentiation qualities, sometimes with an additional application of various growth factors [2]. In the last two decades, mesenchymal stromal cells (MSC) were favored and propagated as cells with an osteogenic differentiation capacity offering osteoinductive [3] and osteogenic [4] components for generating bone tissue. Currently, the differentiation potential of MSC has been optimized to such an extent that MSC are already being used in the first clinical trials for bone regeneration [5]. Given this fact, we focused on the osteogenic differentiation of the underestimated fibroblasts (FB) as they are easy to harvest and proliferate. Moreover, MSC are actually characterized as fibroblastoid cells, which have a phenotype comparable to FB and appear to have more in common than discriminating qualities [6]. The FB are able to differentiate towards several lineages [7] and express most MSC markers. Another reason the FB are a relevant candidate, especially for bone regeneration, is that the genetic profile of the FB is already comparable to the osteoblast's profile [8].

As the request for bone substitutes increases in our aging society, so does the age of the prospective donors for autologous cell transplantation. This is a challenging task, since as the donor age rises, so does the amount of DNA damage, nuclear genome instability, and epigenetic alterations [9] in all somatic cells as well as in FB. Altered pre-mRNA processing, disturbed proteostasis, redox-imbalance, and increased mitobiogenesis are also observed in elderly FB [9]. All these factors potentially affect their differentiation capacity. For these reasons, it is essential to examine the differentiation potential from young in comparison to old donors. Moreover, it is worth taking a closer look at culture senescence of FB as some proliferation steps are necessary to obtain sufficient cell numbers for clinical use. One consensus is, that for useful therapy, at least 1×10^6/kg or 10^8 total cells are required for successful *in vivo* application [10].

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In some instances, it is necessary to increase proliferation with growth factors or cytokines to obtain a sufficient cell number for transplantation. Not only in this context, but also concerning osteogenic differentiation potential the impact of culture senescence is frequently overlooked.

Furthermore, it should be noted that the osteogenic differentiation is commonly initiated by addition of ascorbate-2-phosphate, dexamethasone (dexa), and β-glycerophosphate to standard cell culture medium. Though this protocol may be an adequate method to induce the differentiation of multipotent cells in general, it may not be the most effective protocol for the differentiation of foreskin FB into the osteoblastic phenotype for bone tissue engineering, because the osteogenic differentiation capacity of multipotent cells is dependent on their tissue origin [11].

In this study, we analysed the effect of growth factors and cytokines on early and late-culture passages of FB. Furthermore, the osteogenic differentiation potential of human foreskin FB depending on donor age and culture senescence was evaluated, improved and optimized.

Materials and Methods

Patients

Study approval was obtained from the Ethics Review Board of the Medical Faculty, Heinrich- Heine-University Düsseldorf (Study No. 3634). The age of the male donors was separated into either "young" donors up to the age of 15 years (age: 1-15; in total 21 donors; median=4.52 years) or "old" from the age of 22 years (age: 22-76; in total 11 donors; median=58 years). Distinguishing criterion was the assumed concluded puberty. The specific numbers used are indicated in figure captions. All donors were analysed separately and were not pooled. All patient-related data were anonymised for analysis. Unless specified otherwise, all chemicals and cell culture materials were obtained from Merck KGaA, Darmstadt, Germany. The usage of human material was approved by the local ethics committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf (study number 3634) and conducted in compliance with the Declaration of Helsinki Principles. Informed written consent was gathered from all patients.

Isolation of FB

Foreskin FB were isolated from skin tissue as described previously [7]. Briefly, skin samples were cut into small pieces and digested overnight with 0.2% dispase II-solution. The samples were then treated with 0.2% collagenase buffer (Type: CLS 255 U/mg) (1 mM CaCl, 5 mM glucose, 0.1 M HEPES, 0.12 M NaCl, 50 mM KCl in aqua dest.) for 2 h at 37°C in a shaking water bath to release the cells from the tissue matrix. Following the digestion step, the suspension was passed through a 100 µm filter, washed with PBS, and centrifuged at 800 × g for 5 min (21°C). The cell pellet was resuspended in the standard culture medium and incubated at 37°C, 5% CO_2.

Standard cultivation of FB

Standard cultivation medium comprising DMEM (4.5 g/l glucose), 2 mM α-glutamine with 10% FBS (FCS; Atlas biological, CO, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin was used. 3.75 x 10^5 cells were cultured in 75 cm^2 culture flasks, or cells were seeded in 6- or 24-well-plates, and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Antigen phenotype characterization

The antigen phenotype of FB was characterized by a fluorescence-based flow cytometer (FACSCalibur analyzer and Cell Quest Software, Becton Dickinson Biosciences, Heidelberg, Germany) and used conjugated antibodies against CD44, CD73, CD45, CD90, CD34, HLADR, CD13, CD26, CD105, CD14, CD29, and CD19 (Becton Dickinson Biosciences, Heidelberg, Germany). An appropriate isotype-matched control antibody was used as a control in all the analyses. FACS-analysis was performed with cells in P ≤ 3 versus culture senescent cells in P>3 (old). For analysis, the respective cells were detached with 0.5% trypsin/0.02% EDTA, washed, centrifuged at 200 × g for five min, and reestablished on ice for 15 minutes. The cells were then centrifuged at 200 × g for five min, re-suspended in CellWash® (Becton Dickinson Company) containing 3% FBS, and stained for 30 minutes with the fluorophore-conjugated antibodies. After another washing step with CellWash/FBS, the samples were analyzed.

Application of cytokines and growth factors

Cells were seeded in 3 × 10^3/cm^2 After 48 h, 72 h, and 96 h, cells were treated with cytokines at concentrations from 1 to 1000 U/ml or in the case of Deta-NO from 5 µM to 100 µM. After 120 h, cell proliferation was assessed with the CellTiter-Blue® assay.

Stimulation with FGF-2

FB were seeded in ascending cell numbers from 1 to 10 × 10^3. After 24 h, a single treatment of FGF-2 was performed, or FGF-2 was applied daily at a concentration of 333 U/ml. On day 5, the cell proliferation was analysed with CellTiter-Blue®.

CellTiter-Blue® assay

Following the manufacturing protocol, cell viability was determined by an 1 h incubation with CellTiter-Blue® (Promega, Mannheim, Germany) used at a 1:20 dilution in medium. The obtained fluorescence signals were proportional to the number of viable cells and were analyzed with the Elmer Victor 2 plate reader at 560_{EX} /590_{EM}.

Osteogenic differentiation medium

Osteogenic differentiation medium (OM) consisted of standard cultivation medium supplemented with dexamethasone (dexa; 100 nM), α-ascorbin-2-phosphate (50 µM), and β-glycerophosphate (10 mM) [12]. Additionally, the OM was supplemented with 5′- Azacytidine (1 µM), or in the case of calcitriol-medium, dexa was replaced by 10 nM 1α,25 dihydroxycholecalciferol (stored and used in darkness). Furthermore, osteogenic differentiation was induced by adding standard cultivation medium supplemented with dexa (1-100 nM), α-ascorbin-2-phosphate (30 µM), β-glycerophosphate (10 mM), or insulin-like growth factor-1 (IGF-1) (100 ng/ml) on collagen type 1 6-well-plates. Osteogenic differentiation was started, when cells are confluent and medium was replaced twice a week.

Effect of pH-value

FB were differentiated towards the osteogenic lineage using OM for 21 days. To evaluate the effect of the pH-level on differentiation potential, FB were exposed to the surrounding air (0.05% CO_2) for 10 min daily. To measure the effect of humidified atmosphere at 37°C, 5% CO_2 on OM pH, OM was placed inside the incubator at 37°C for 1 h and pH was analysed.

Osteogenic differentiation with dexa and calcitriol

Initially, foreskin FB were differentiated with OM for two weeks. Afterwards, dexa was replaced by 10 nM 1α, 25-dihydroxycholecalciferol, and FB were treated with this medium for another two weeks. Twice a day, cell cultures were shaken for 5 min at 75 r/min to induce shear stress.

Determination of ALP activity

Cells were washed with PBS and covered for 10 minutes with 10 mM 4-nitrophenolsolution. The resultant changes in solution absorbance were quantified using a photo-spectrometer at 450 nm (Perkin Elmer Victor 2 plate reader). The absorbance values obtained were normalized to the respective sample using the CellTiter-Blue® assay.

Determination of protein concentration

Protein concentration was determined with a Pierce BCA Protein Assay Kit, following the manufacturer's instructions.

Alizarin red S staining

To determine the presence of extracellular calcium deposits secreted after osteogenic differentiation, the cell cultures were washed with PBS, fixed with 4% (v/v) paraformaldehyde for 15 min, rinsed 2 times with PBS, covered for 20 min at 37°C with 1 ml alizarin red S (0.5% in aqua dest., pH=4.1), and finally washed twice with dH20 [13]. The stained cells were documented by phase contrast microscopy (Zeiss Axiovert 200 microscope). To quantify the amount of alizarin red S incorporated in the calcified matrix, the stained samples were incubated with cetylpyridinium chloride (10% in 10 mM sodium phosphate, pH=7.0), and the extracted alizarin S dye was quantified by analysing the absorbance at 600 nm [14].

RNA extraction from cultured cells

RNA was extracted using TRI Reagent after 10-20 sonification steps. Following an incubation time of 5 min at room temperature (21°C), chloroform was added, and the samples were vortexed. The phases were separated and centrifuged (11,200 rpm for 15 min at 4°C). The colorless phase was taken and mixed with isopropanol. After incubation for 10 min and centrifugation (11,200 rpm for 10 min at 4°C), the pellet was resuspended in 75% EtOH. Then, the samples were centrifuged (8,800 rpm for 7 min at 4°C), EtOH was removed, and the cells were resuspended in RNAse-free water. RNA was purified with a DNA-free KIT (Ambion) according to the manufacturer's instructions.

PCR of BMP 6, Osterix, RunX2 and GAPDH

RNA (500 ng) was reverse transcribed to cDNA using RT-Puffer, d-NTPs, RNase-Inhibitor, Omniskript RTase, and RNase-free water. Gene-specific primer pairs are indicated in Table 1. Relative gene expression was measured with ABI Prism 7300 according to the manufacturer's protocol with the following cycling conditions: 95°C, 10 min (1 cycle); 95°C, 15 s, 60°C, 60 s (40 cycles); 4°C hold with SYBR Green (Applied Biosystems) using the fold change ΔΔCT method. The control used is the untreated control on day 1. All the samples were run in triplicate. Expression of each target gene was normalized to the housekeeping gene glycerinaldehyde-3-phosphate- dehydrogenase (GAPDH).

Statistical analysis

Statistical analyses were performed with a one-way ANOVA and a post hoc Bonferroni analysis was used. Data were expressed as mean value and standard deviation (SD). The level of significance was $P \leq 0.05$ and designated with asterisks.

Results

Phenotypic analysis of FB

Interestingly, when $P \leq 3$ was compared with P>3, it could be determined that the expression of antigens CD73, CD90, CD105, CD44, CD13, CD26, and CD29 was significantly increased in the older passage (≥ 95%), and the expression was more consistent with progressive culture senescence is shown in Figure 1A. It could be evaluated, that the antigen expression, as postulated by the International Society for Cellular Therapy, as criterion for MSC rose over the course of passaging. The lack of expression of specific surface antigens such as CD45, CD14, CD34, CD19, and HLADR (≤ 2%) was in line with the requirements, in early passages as well as in culture-senescent cells in young donors.

The optimal seeding density

Proliferation potential from young donors is significantly increased compared to that from old donors. It could be determined that seeding density of 1×10^4/6-well/ (9.03 cm2) showed a stronger proliferative potential (Figure 1B and 1C), because cell count quadrupled in contrast to 1×10^5/6-well, where cell number only doubled.

The optimal growth factor concentration

It could be determined, that in general the FB in early passages ($P \leq 3$) were rather insensitive to the applied growth factors, while the senescent ones were sensitive (P>3). Concentrations of 10-100 units/ml IL-1β elevated the proliferation of FB (P>3) (Figure 2A). The most efficient concentration to improve proliferation with IL-6 was 33 units/ml (P>3) (Figure 2B). To enhance the proliferation with IL-8, 3 units/ml or 100 units/ml (P>3) were needed (Figure 2C). In general, treatment with TNF-α has no distinguishable effect on the growth rate (Figure 2D). If the senescent FB were treated with 1 unit/ml or 3 units/ml IFN-γ, proliferation was significantly increased, compared to early passages. However, a concentration of 10 units/ml IFN-γ or more reduced proliferation of FB in early passages ($P \leq 3$) (Figure 2E). Incubation with either 1 unit/ml or 1,000 units/ml TGF-β in FB (P>3) resulted in a slightly enhanced cell number (Figure 2F). Using 5 μM and 50 μM Deta-NO increased the proliferation of FB (P>3). Particular attention should be paid to the significant difference between the proliferation of FB in early passages compared to the senescent FB. A concentration of 100 μM Deta-NO reduced proliferation in FB in $P \leq 3$ and P>3 (Figure 2G). Finally, the proliferation of FB (P>3) with FGF-2 was most increased by a concentration of 333 units/ml (Figure 2H). Interestingly, culture senescent cells (P>3) are more sensitive to growth factor treatment than FB $P \leq 3$ (Figure 2A-2H), and in $P \leq 3$, FB growth was inhibited by adding TGF-β, IFN-γ, and Deta-NO.

Single versus daily application of FGF-2

To obtain sufficient cells for clinical use, it might be necessary to expand foreskin FB to the maximum possible. In this case, it could be useful to seed even smaller cell numbers, especially for the strong mitogen factor FGF-2. The optimal seeding density for such a proliferating growth factor in P>3 was 2.5 or $3 \times 10^3/cm^2$, dependent on the frequency of growth factor application, because a daily application

	Forward	Reverse
Osterix	GCGTCCTCCCTGCTTGAG	CCAGAGTTGTTGAGTCCCGC
BMP6	ACATGGTCATGAGCTTTGTGAA	TGAACTTCTTTGTGGTGTCGCT
RunX2	TGGGCTTCCTGCCATCACCGA	GGTGGCTGGATAGTGCATTCGTGG
GAPDH	CCCGCTTCGCTCTCTGCTCCT	TGACCAGGCGCCCAATACGAC

Table 1: Primer pairs and sequences.

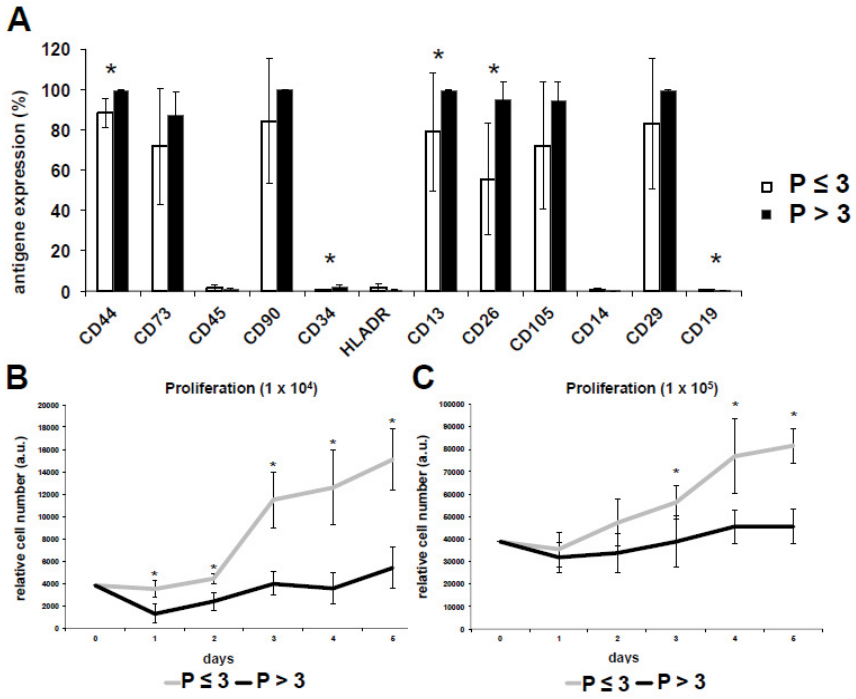

Figure 1: Analysis of the phenotype and the proliferation rate of human FB from young donors. **A** is Antigen representation of FB in early passages (P ≤ 3) and culture senescent passages (P>3). Antigen expression of CD44, CD13, CD26, CD29, CD73, CD90, CD105 were increased in later passages. (n=19) **B** Evaluation of the proliferation of FB 1 × 10^4/Well in P ≤ 3 and P>3. The proliferation of early passages of FB was significantly increased compared to the later (n=8). **C** Analysis of the proliferation of FB seeded in different cell numbers in P ≤ 3 and P>3. FB seeded in a cell density of 1 × 10^4/6-Well in P ≤ 3 had a stronger proliferative potential (Figure 1B and 1C; n=8).

of 333 units/ml FGF-2 has an advantage over a single application concerning the proliferation (Figure 2I).

The optimal passage

To evaluate the osteogenic differentiation potential, the alkaline phosphatase assay was performed. It could be elucidated, that the young and early passages of FB had expressed significantly more active alkaline phosphatase in a period of differentiation (day 7-21) (Figure 3B). Moreover, it could be demonstrated, that the deposition of calcium as further evidence for improved osteogenic differentiation was almost five times higher in young FB upto P ≤ 3 compared to all other groups on day 21, which is significant. To establish whether increased protein concentration was responsible for the evaluated osteogenic differentiation potential, protein concentration was analysed. As shown in Figure 3C, the protein concentration is the highest in young FB at later passages (P>3), which invalidated this assumption. When compared in detail, P 3 is the optimal passage to differentiate osteogenetically, rather than P 1 (Figure 3D). Although the enhancement is not significant, the later passage is to be preferred, because cell number is higher in later passages and this is an advantage for a clinical use.

Dexa for osteogenic differentiation

When OM, the standard differentiation medium, consisting of DMEM (4.5 g/l glucose), 2 mM α-glutamine with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin with α-ascorbin-2-phosphate (50 µM) and β-glycerophosphate (10 mM) was used, the optimal dexa concentration to improve osteogenic differentiation, considering calcium deposition of foreskin FB was 100 nM (determined after 21 days) (Figure 4).

Effect of pH-value

If OM was exposed to 5% CO_2 in the incubator (37°C) for one hour, the pH value was significantly decreased (Figure 5). Furthermore, it could be demonstrated that higher pH values reduced the deposition of calcium in the extracellular matrix and the alkaline phosphatase activity as demonstrated in Figure 5, due to repeated exposures to 0.05% CO_2.

Treatment with 5′Aza

Treatment with 5′Aza had no supporting effect on the osteogenic differentiation of foreskin FB (Figure 6A-6C).

Application of calcitriol

When calcitriol was used instead of dexa for the osteogenic differentiation, FB failed to deposit calcium in the extracellular matrix (Figure 6A). Interestingly, the enzymatic activity of alkaline phosphatase is increased compared to OM. However, it is important to note that the alkaline phosphatase activity was comparatively high from the beginning (Figure 6B; day 0-14). At day 21, the OM medium is significantly superior to the calcitriol medium, concerning protein expression, alkaline phosphatase activity and calcium deposition (Figure 6D-6F). When the experimental setting was modified in such a way that calcitriol was only used after a 14-day treatment with OM (with dexa) and the FB were also exposed to shear stress twice a day throughout this period, osteogenic differentiation could be improved. So, calcium deposition on day 21 is significantly higher in these calcitriol-treated FB compared to control cells, and alkaline phosphatase activity was comparable (Figure 6G-6I).

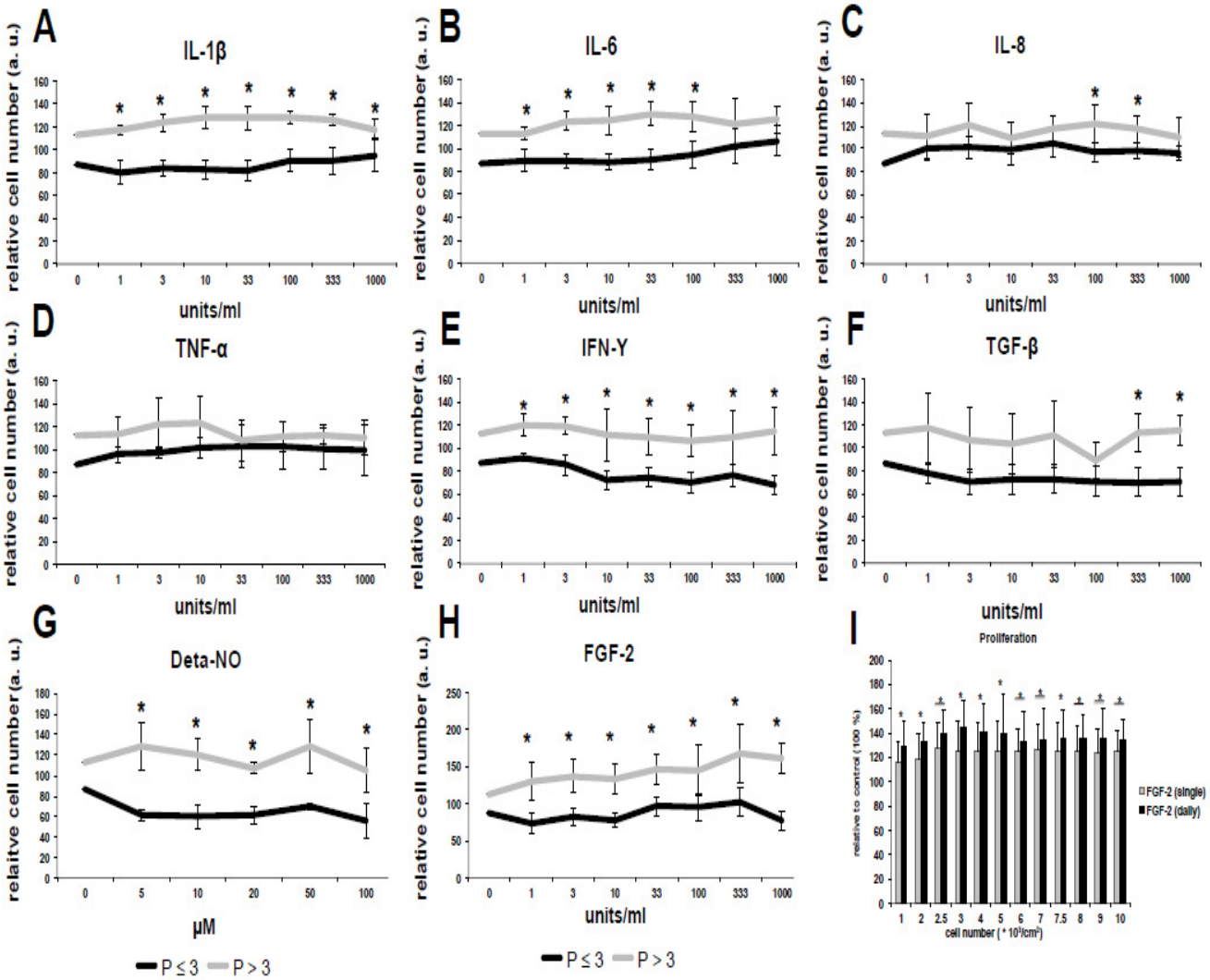

Figure 2: Impact of diverse cytokines and growth factors on proliferation of FB in P ≤ 3 and P>3. The analysis was performed 5 days after seeding in young donors**.** The mean value of the untreated control of P ≤ 3 and P>3 was set at 100 and all other values were calculated in relation. **A** is the effect of IL-1β. An application of 33 units/ml IL-1β enhanced the proliferation up to 30% in culture senescent FB compared to FB in early passages. **B** The effect of IL-6. An application of 33 units/ml IL-6 enhanced the proliferation up to 30% in culture senescent FB compared to FB in early passages. **C** is the effect of IL-8. The strongest effect on proliferation had an application of 100 units/ml in P>3, and this effect differed approximately 20% from P ≤ 3. **C** The effect of TNF-α 3 or 10 units/ml TNF-α had the strongest effect on growth rate of P>3. In P ≤ 3 an application of 33 or 100 units/ml TNF-α had the strongest effect on proliferation. **E** The effect of IFN-γ. IFN-γ had little influence on proliferation of early or late passages. However, values differed significantly from each other **F** The effect of TGF-β. TGF-β had no increasing effect on the proliferation of FB in P ≤ 3 and P>3. On the opposite in FB in P ≤ 3 proliferation was inhibited. **G** The effect of Deta-NO in concentrations of 5 or 50 µM Deta-NO could increase proliferation up to 10% compared to the respective control P>3. In P ≤ 3 Deta-NO inhibited the growth rate in all applicated concentrations. **H** The effect of FGF-2. FGF-2 had the strongest effect on the proliferation of FB in passages>3, up to 50 % compared to the respective control. All applicated concentrations differed significantly from the respective FB in early passages (P ≤ 3). **I** is evaluation of seeding density and comparison of a single or a daily application of FGF-2. For a strong mitogen factor such as FGF-2 a seeding density of 2.5 or 3 × 10^3/cm^2 was optimal and a daily application of FGF–2 (333 units/ml) is a superior to a single treatment. (n=5) * P ≤ 0.05, bars represent standard error +/- (SE) of individuals experiments indicated.

Osteogenic differentiation with IGF-1

Already on day 7 the osteogenic differentiation with IGF-1 is significantly superior to the standard differentiation medium. From day 14 forward osteogenic differentiation of FB treated with IGF-1 seeded on collagen I was enhanced in comparison to OM medium (Figure 7A). Only in these set up conditions collagen I was used, because the additional treatment with IGF-1 resulted in cell detaching in the course of osteogenic differentiation (data not shown).

mRNA expression of BMP6, RunX2, and Osterix

When FB were differentiated osteogenically with OM and IGF-1 on collagen I-coated tissue culture plates, the mRNA expression levels of BMP6, RunX2, and Osterix (Figure 7B-7D), essential for osteogenesis, were increased over the course of time.

Discussion

Bone is the second most transplanted tissue. More than 2 million

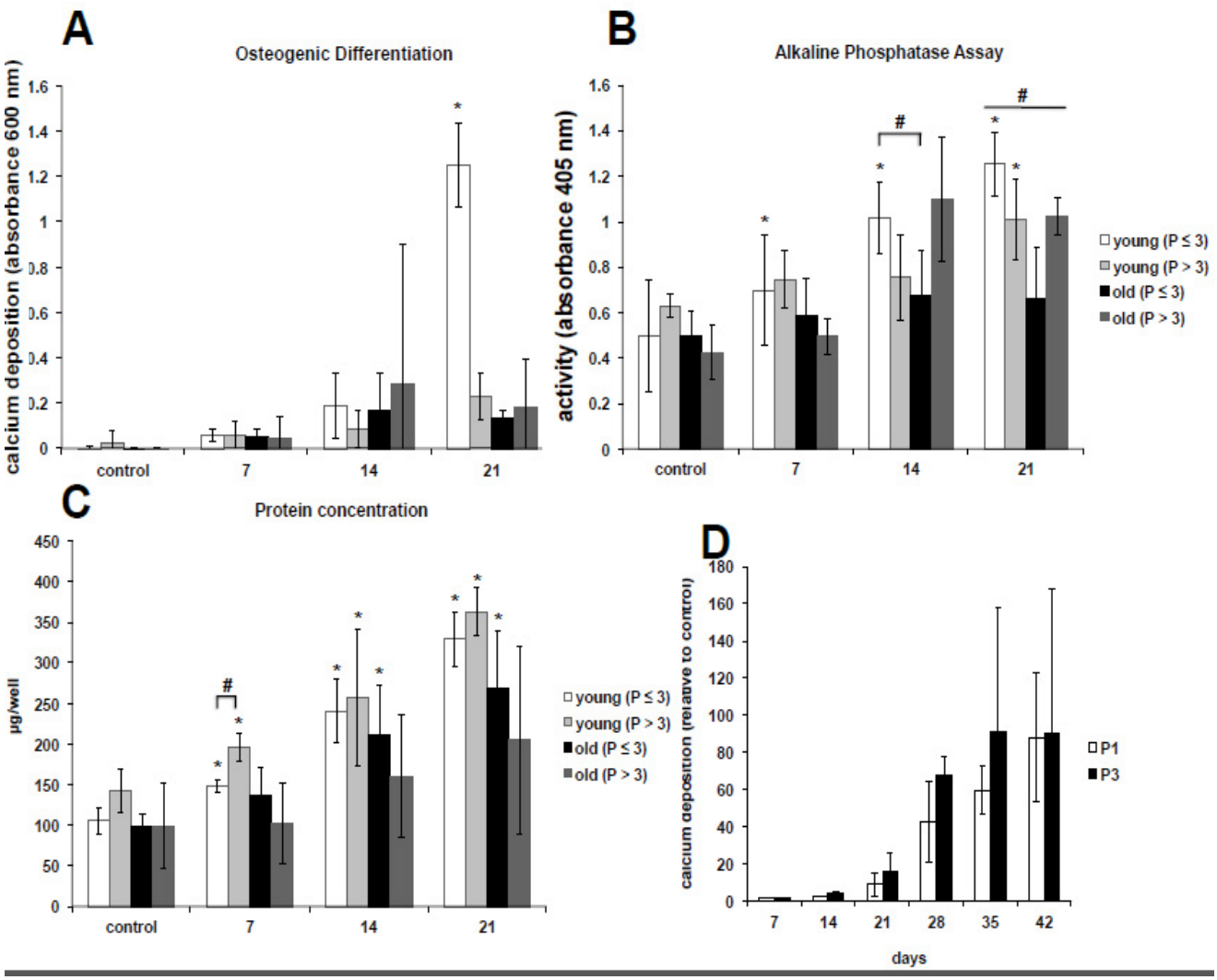

Figure 3: Comparison of the osteogenic differentiation potentials of young versus old donors and early versus late passages. In Figure 3, **A** is evaluation of osteogenic differentiation potential with alizarin red S. FB from young donors up to passage 3 (P ≤ 3) were significantly superior compared to all other groups (approx. six times). **B** is analysis of the alkaline phosphatase assay. FB from young donors in P ≤ 3 were significantly elevated in their capacity to express an active phosphatase in the course of the osteogenic differentiation, particularly on day 21. **C** is measuring protein concentrations in the process of osteogenic differentiation. Highest protein concentration was determined on day 21 FB from young donors P>3. **D** Comparison of osteogenic differentiation potential of passage 1 (P 1) and passage 3 (P3) in young donors. (n=4) * p ≤ 0.05, bars represent standard error +/- (SE) of individuals experiments indicated.

bone transplantations per year are conducted worldwide [15] and due to the demographic development, non-healing bone defects as a consequence of trauma, tumours, pseudoarthrosis, and osteoporosis will further increase. The current gold standard to treat critical size defects is the application of the autologous spongiosa [16], but donor site morbidity and limited availability has compelled researchers to explore promising alternatives. FB have been considered a powerful tool for the regeneration of bone tissue because they are one of the easiest cell types to process and to expand *in vitro* [7] with mesodermal, ectodermal, and endodermal differentiation potential [17,18]. FB also have immunomodulatory features like MSC [19], which predestine them for use in cell-based therapies.

For the implementation of FB or autologous cells in a therapeutic application, some proliferation steps are usually necessary. Moreover, the surgical procedure for cell- withdrawal should be kept to a minimum to avoid patient inconveniences and infection risks. Therefore, the aim is to expand a minimum of cells to a sufficient number to heal even large (bone) defects. One strategy is to increase the cell number by passaging several times, thereby accepting culture senescence. In this study, the phenotypical changes in early (P ≤ 3) versus late-culture passages (P>3) of foreskin FB were evaluated. Interestingly, our data determined that the antigen expression of CD73, CD90, and CD105, defined by Dominici et al. [20] as minimal criteria of multipotent MSC, was improved over multiple passages. The percentage of FB stained positive for each of these markers significantly increased up to ≥ 95% in later passages. The expression of CD13, CD26 [21] CD44, and CD29 was also in parts significantly enhanced in culture-senescent FB (Figure 1A). It could be assumed that culture-senescent FB had a more homogenous profile with high levels of stromal markers, as it was observed by Mitchel et al. for adipose-derived stromal cells (ASC) [22].

Cellular senescence limits the proliferation of cells [23]; therefore, it

is not surprising that FB in earlier passages have a proliferation capacity twice as high as culture-senescent FB (Figure 1B and 1C). Nevertheless, it was astonishing that with an optimal seeding density of 1×10^4/6-Well (Figure 1B), the number of proliferated cells could be nearly quadrupled.

Another strategy to induce proliferation is to use cytokines as IL-1β [24], IL-6 [25], IL-8 [26], TNF-α [27], IFN-γ [28], and growth factors as TGF-β [29], FGF-2 [30], or Deta-No [31] as nitric oxide donor to increase cell number. In this context, the impact of culture senescence is often ignored. Note worthily, as shown in (Figure 2), some growth factors had completely different effects depending on the concentration and the passage of FB. The proliferation of FB in cell culture passage $P \leq 3$ was hardly affected by IL-1β, IL-6, IL-8, and TNF-α (Figure 2A-2D) in comparison to older passages P>3 where proliferation could be significantly increased compared to the initial cell number. Treatment with 1-1,000 units/ml IFN-γ had elevated proliferation in P>3 significantly, and best results were accomplished with 10 or 1,000 units/ml (Figure 2E). However, only high amounts (333+1,000 units/ml) of TGF-β could propagate cell numbers in P>3 (Figure 2F). Similar observations were made with Deta-NO. While an application of 5 μM or 50 μM Deta-NO induced proliferation in older passages (P>3), it reduced proliferation in early passages ($P \leq 3$) (Figure 2G). Surprisingly, 1 unit/ml FGF-2 slightly inhibited proliferation in $P \leq 3$; in P>3, proliferation was strongly induced (Figure 2H). However, FGF-2 increased proliferation the most, and treatment with 333 units/ml was determined to be the ideal concentration. Hence, we analysed this result in detail. It could be determined that daily application of FGF-2 to FB in connection with a seeding density of $3 \times 10^3/cm^2$ was optimal for maximum proliferation in later passages (P>3).

For using autologous cells in regenerative medicine, the age of the donors must be considered. Therefore, in addition to comparing the osteogenic differentiation potential of early passages ($P \leq 3$) versus culture-senescent FB (P>3), the differentiation potentials of young versus old-donor FB were analysed and significant discrepancies were determined (Figure 3A-3C). FB from young donors in early passages were significantly superior in their osteogenic differentiation potential as evaluated by quantification of calcium deposition (Figure 3A). Similar findings were made investigating the expression of alkaline phosphatase as an indicator of the osteogenic differentiation potential; again, young FB in early passages $P \leq 3$ were significantly predominant (Figure 3B). Lastly, to evaluate the best passage for differentiation, P 1 was compared to P 3, and it was determined that FB from young donors in P3 were optimal to differentiate osteogenically (Figure 3D). In accordance with these results, henceforth foreskin FB in P3 were used to examine and improve the osteogenic differentiation potential.

The optimal dexamethasone (dexa) concentration was determined as dexa increases the expression of RunX2 [32], a crucial factor for osteogenic differentiation. The dexa concentration also has significant effects on mineralization and differentiation as shown by Mikami et al. [33]. Similar to what Mikami et al. demonstrated for chick periosteum cells, our results suggest, that 100 nM dexa was the optimal concentration for osteogenic differentiation of human FB (Figure 4).

Bone remodeling is also affected by ionic and molecular components of the extracellular matrix in which calcium phosphate salts are precipitated in a pH-dependent manner [34]. There are indications that an alkaline environment may facilitate the mineralization of osteoblasts [35,36], whereas acidic surroundings can impair osteoblast differentiation and can cause osteoblast death [37-39]. Even a short time outside the incubator led to a raised pH of the osteogenic differentiation medium. Therefore, we analysed the osteogenic differentiation potential while exposing FB to the surrounding air (CO_2 0.05%) daily, as the pH of the osteogenic differentiation medium is enhanced from 7.5 to 8 in only one hour under ambient air conditions (Figure 5D). Surprisingly, the osteogenic differentiation potential of foreskin FB was impaired (Figure 5A-5C) by increasing the pH value under these conditions. Therefore, our findings contradict the results obtained by Fliefel et al., who determined that pH=8 was ideal to induce matrix calcification in human MSC [34]. The reduction of osteogenic differentiation potential noted in the present study might be, because the FB were affected by the ambient temperature (21°C-23°C), which was notably lower than the incubators, as observed by the working group of Chen et al. [40].

DNA methylation has been characterized as a regulatory mechanism of genome function, mediator of embryonic development, imprinting, X-chromosome inactivation, chromosomal stability, transcription, and differentiation [41]. Several genome-wide studies show that DNA methylation patterns are person-specific, tissue-specific, and furthermore dependent on localization of the tissue [42]. Initial successes could be accomplished differentiating aged ASC into the osteogenic [43] or hepatic [44] lineage using 5′Aza, because 5′Aza induces DNA demethylation by inhibiting DNA methyltransferase [45] and can re-activate previously silent genes [46]. In this study, 5′Aza was used to determine if tissue- or person- specific methylation patterns lower osteogenic differentiation potential in foreskin FB. However, as shown in (Figure 6A and 6B) no significant elevation of osteogenic differentiation potential could be observed in FB additionally treated with 5′Aza. A permanent application of 5′Aza is potentially contraindicated, as it is possible that tissue- and person-specific methylation patterns will be removed, but critical osteogenic differentiation-specific methylation patterns will be impeded. However, methylation patterns might play a subordinate role, using young FB from young donors in P=3, as demonstrated in ASC from donors younger than 45 years [43].

Another attempt to improve osteogenic differentiation potential was to utilize the active form of the vitamin D metabolite 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), also known as calcitriol, as a substitute for dexa. Calcitriol assumes an important role in skeletal homeostasis, since calcitriol controls bone metabolism by modulating osteoblasts and osteoclasts in bone tissue directly, and additionally anabolic as well as catabolic effects have been demonstrated on osteoblast differentiation and bone formation [47]. These findings are supported by *in vitro* studies reporting that calcitriol can induce expression of both early as well as late markers of osteoblast differentiation in human MSC, including osteocalcin, osteopontin, bone sialoprotein, and alkaline phosphatase [47-49]. Furthermore, it was shown that calcitriol enhances dexa-induced osteogenic differentiation of human pre-osteoblasts and MSC, resulting in an increased activity of alkaline phosphatase and elevated matrix calcification [47-49].

Our data illustrates, that only applying calcitriol (and α-ascorbin-2-phosphate, β-glycerophosphate) for osteogenic differentiation had no beneficial effect on matrix mineralization (Figure 6D). On the contrary, the activity of alkaline phosphatase is increased in tendency (Figure 6E), as demonstrated in the studies of Beresford et al. [49]. However, it is noteworthy that in this study calcitriol and dexa were deliberately not applied simultaneously, because the osteoinductive effect could have been reciprocally canceled dependent on the concentration of both steroids, as observed in other studies [50-52]. Due to the limited success, sequential application of dexa and calcitriol was performed in subsequent experiments. Given the beneficial results observed with shear stress in

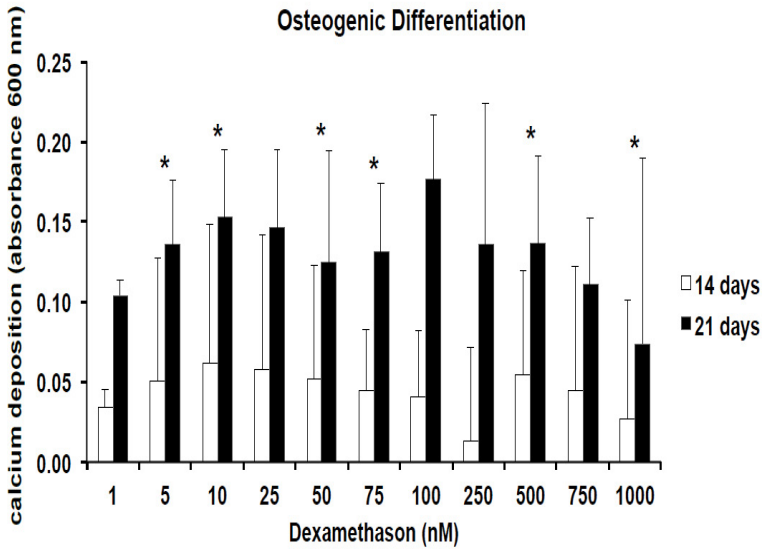

Figure 4: Evaluation of optimal dexa concentration for osteogenic differentiation of FB. Diverse dexamethasone concentrations in the range from 1-1000 nM were used. Analysis was performed on day 14 and 21 in young donors in P ≤ 3. Considering calcium deposition a concentration of 100 nm was the optimal dexamethasone concentration to improve the osteogenic differentiation in this context. (n=3) * p ≤ 0.05, bars represent mean +/- standard deviations (SD) of the individuals experiments indicated. Compared are values from day 14 with day 21.

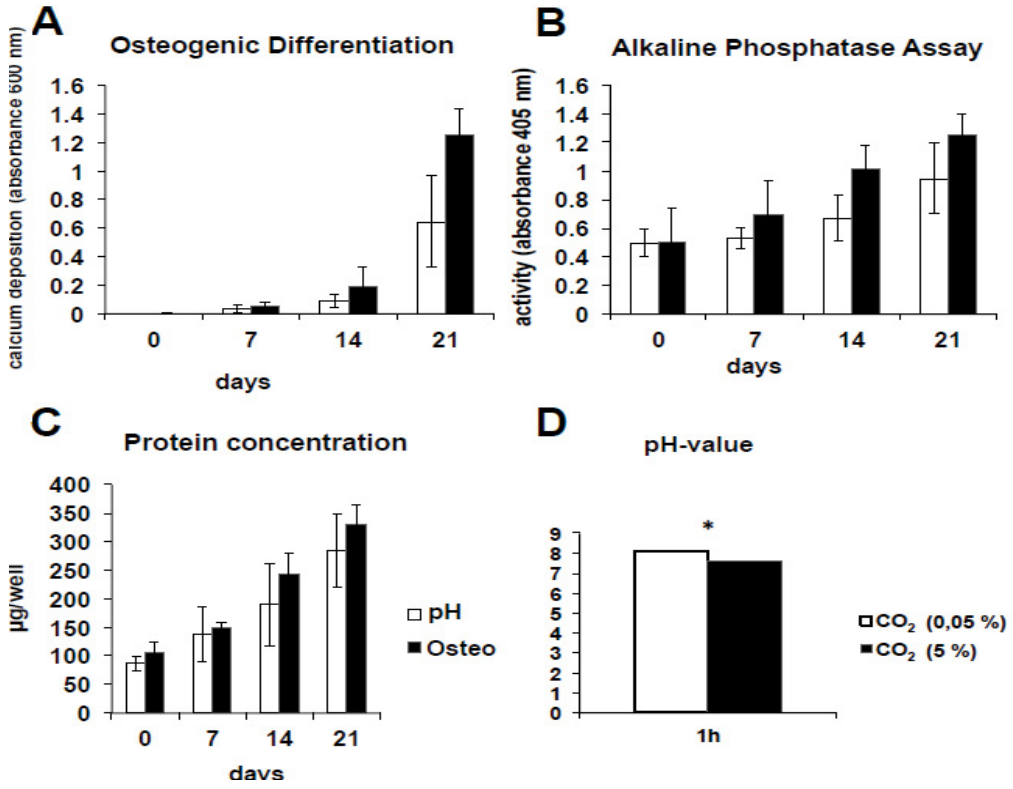

Figure 5: Impact of the pH-value on the osteogenic differentiation potential of FB. **A** is evaluation of osteogenic differentiation potential with alizarin red S in young donors P ≤ 3. **A** Smaller pH-value supported in tendency the capacity to deposit calcium in the extracellular matrix. **B** The potential to express an active alkaline phosphatase seemed to be provided by a lower pH. **C** Measuring protein concentration. Protein concentration of OM was slightly increased in the course of differentiation. **D** The analysis the pH value of OM. After 1 h deposition of OM at 0.05% CO_2 pH value was significantly increased. (n=6), * p ≤ 0.05, bars represent standard error +/- (SE) of individuals experiments indicated.

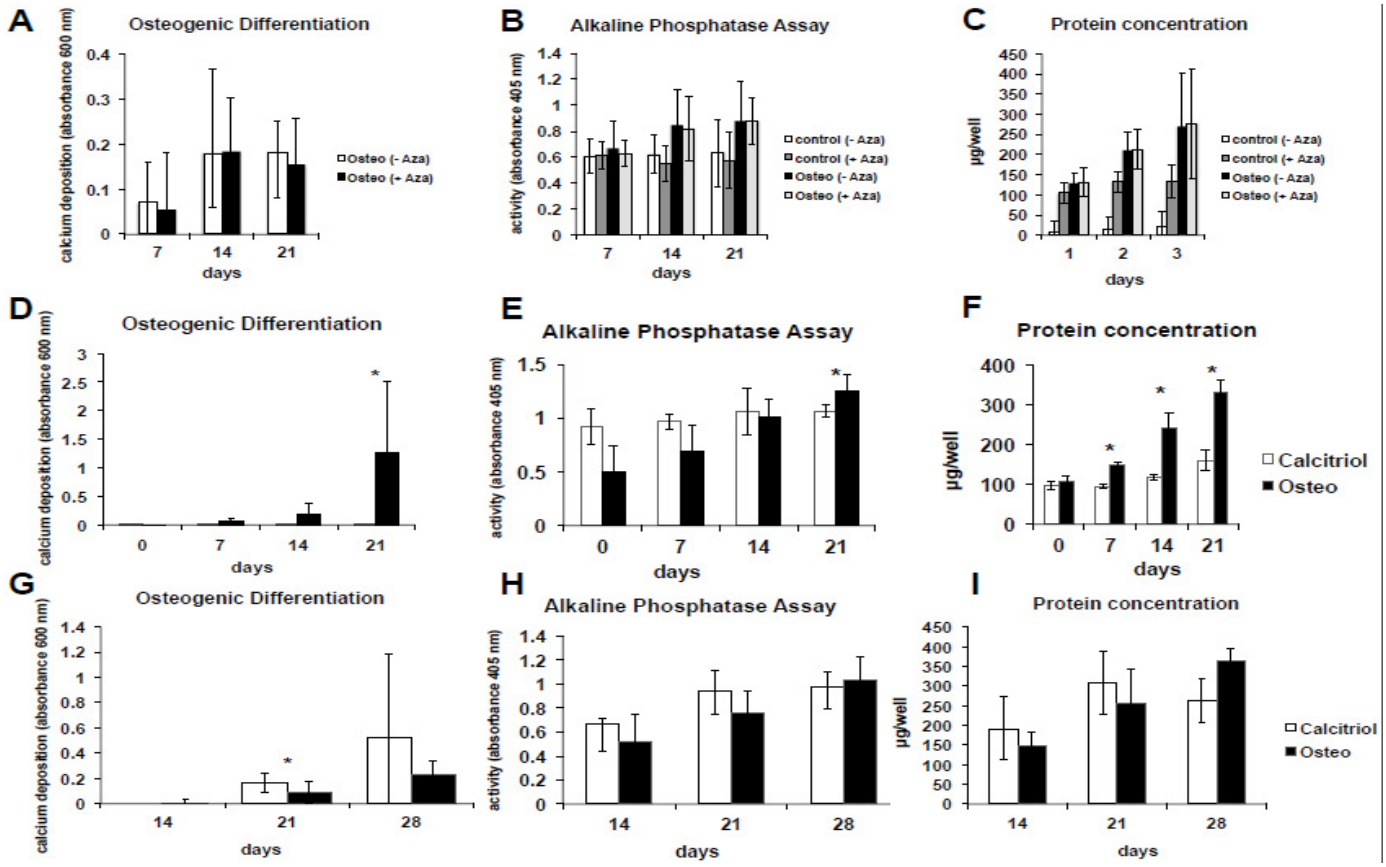

Figure 6: Analysis of the osteogenic potential of of 5′ azacytidin (Aza) or Calcitriol in young donors P ≤ 3. **A** is impact of Aza on the osteogenic differentiation potential analysed with alizarin red S. An additional Aza supplementation to OM had no beneficial effect on the calcium deposition. **B** is the alkaline phosphatase activity could not be increased with Aza, Aza+OM compared to cells compared with standard cell culture media (control +/- Aza.) **C** is azacytdin had no effect on the protein expression in progress of osteogenic differentiated and compared to cells cultivated with standard cell culture media (control +/-Aza). **D** isThe substitutional application of calcitriol instead of dexamethasone in OM inhibited significantly the osteogenic differentiation capacity analysed with alizarinred-S on day 21. **E** is the activity of alkaline phosphatase is significantly inhibited by the treatment with calcitriol only on day 21. **F** is in the course of the osteogenic differentiation calcitriol diminished protein expression significantly compared to OM from day 7. **G** Effect of shear stress and a serial treatment with OM and afterwards calcitriol (n=4). After 28 days serial treatment with OM and calcitriol was superior to a solely incubation with OM. The difference is significant on day 21. **H** Alkaline phosphatase activity was comparable to FB treated with calcitriol from day 14. **I** is protein concentration in FB incubated with OM was in tendency higher than in calcitriol at day 28. (n=4), * p ≤ 0.05, bars represent standard error +/- (SE) of individuals experiments indicated.

dermal FB [53], the FB were additionally shaken twice a day for 5 min at 75 rpm. Finally, the osteogenic differentiation potential could be successfully significantly improved (Figure 6G), as determined by enhanced matrix calcification. Alkaline phosphatase activity could also be increased in tendency (Figure 6H). However, because protein concentration (Figure 6I) was also increased, it is possible that a sequential application of dexa and calcitriol in addition to shaking twice a day improved the number of cells as well.

Our concluding approach succeeded in improving osteogenic differentiation potential with insulin-like growth factor 1 (IGF1), α-ascorbin-2-phosphate, β-glycerophosphate, and dexa. IGF1 was used, because it is one of the most available growth factors in bones, and with the corresponding receptor (IGF1R), it constitutes a growth-promoting signaling system for the skeleton [54]. *In vivo* circulating IGF1 is produced in the liver and has a role in bone remodeling, but skeletal IGF1 production is likely to play the major role [55], since it modulates osteoblastic proliferation, differentiation, survival, and the synthesis of bone matrix [56,57]. Therefore, we made the attempt to increase osteogenic differentiation potential of foreskin FB with standard cultivation medium supplemented with dexa (100 nM), α-ascorbin- 2-phosphate (30 µM), β-glycerophosphate (10 mM), and IGF1 (100 ng/ml) on collagen type I- coated wells. As shown in Figures 7 and 8, this strategy performed the best. Significant difference could be determined at day 7, starting with day 14 the cells supplemented with IGF1 differentiated better osteogenically. To determine, if accelerated osteogenic differentiation potential corresponds with an induction of genes specific for osteogenic differentiation, the expression of Osterix, RunX2, and BMP-6 was analysed. Osterix and RunX2 are two indispensable transcription factors considered the master regulators of osteogenesis [58]. BMPs were shown to be important in osteoblast differentiation and bone formation *in vivo*, where they induced differentiation and synthesis of extracellular matrix with other cytokines. Furthermore, it was demonstrated that an administration of BMP-6 induced new bone formation [59] and its expression was enhanced *in vitro* and *in vivo* by IGF1 [59]. As demonstrated in Figure 7B-7D, these factors could still be increased over the course of osteogenic differentiation.

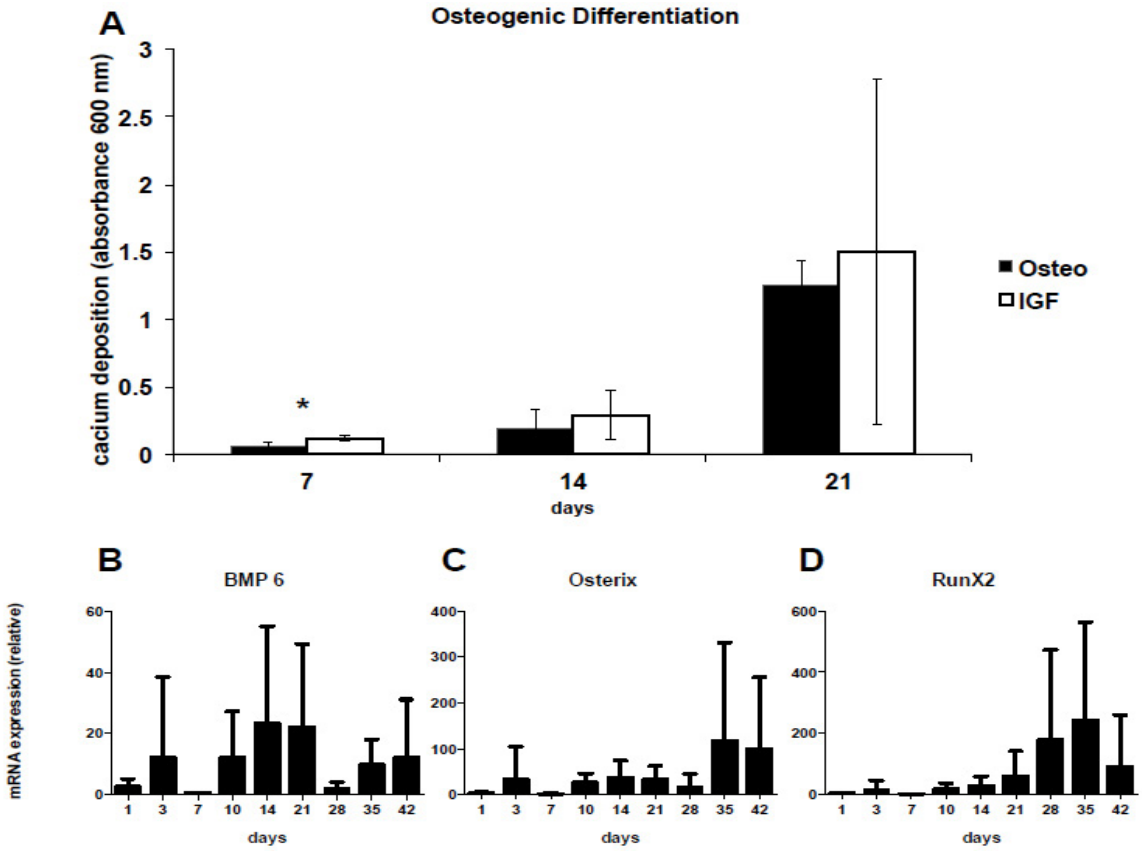

Figure 7: Impact of IGF-1 and collagen I on the osteogenic differentiation of FB in young donors P ≤ 3. **A** is analysis of osteogenic differentiation measured with alizarin red S relative to the untreated control. At day 7 osteogenic differentiation was significantly raised in FB treated additionally with IGF-1 on collagen I compared to the cells differentiated with OM (*). **B** In young FB (P=3) osteogenic differentiated with IGF and collagen I BMP-6 mRNA expression was raised in tendency in the course of time. **C** is in young FB (P=3) osteogenic differentiated with IGF-1 on collagen I Osterix mRNA expression was elevated in tendency from day 35 in the course of osteogenic differentiation. **D** is in young FB (P=3) osteogenic differentiated with IGF on collagen I RunX2 mRNA expression was tended to increase from day 21 on. (n=8), * p ≤ 0.05, bars represent standard deviations +/- (SD) of individuals experiments indicated.

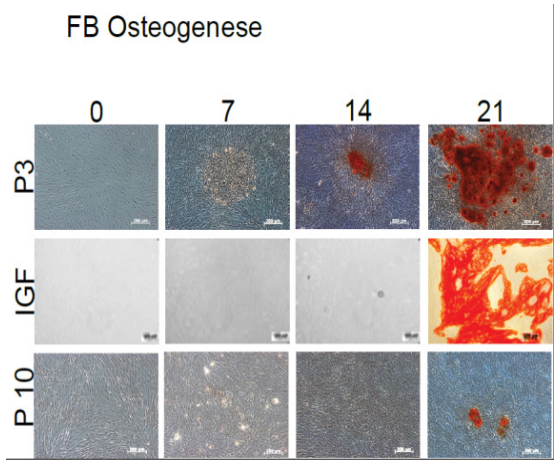

Figure 8: Visualized comparison of osteogenic differentiation potential of FB from young donors in P 3, P 10, and P 3 treated with IGF-1 on collagen I. Evaluation was performed with alizarin red S on day 0, 7, 14 and 21. Shown is one representative illustration of six identical results as a minimum. Graphic bars represent a length of 200 µm.

The present study demonstrates the impact of donor age and cellular senescence on proliferation and osteogenic differentiation potential as well as how the osteogenic differentiation capacity of human FB could be improved. Several key findings were made for the application of foreskin FB in cell-based therapies for tissue regeneration. The cytokines and growth factors we tested had no elevating effect on proliferation in early cell culture passages P ≤ 3; solely the cell number could be increased using optimal seeding density. For bone tissue engineering, only FB from young donors in early culture passages P ≤ 3 are beneficial, as it was observed for skin FB differentiated to the hepatic lineage [60].

Importantly, osteogenic differentiation potential of FB could be significantly improved with additional IGF1 supplementation. In conclusion, it could be demonstrated that FB from young donors in cell culture passage 3 are viable alternatives and useful tools for bone tissue engineering.

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