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Tissue based strategies for bone regeneration: fat, muscle and fascia

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Publication 1

Osseous differentiation of human fat tissue grafts: From tissue engineering to tissue differentiation

Maryna Bondarava, Chiara Cattaneo, **Bin Ren**, Wolfgang E. Thasler, Volkmar Jansson, Peter E. Müller & Oliver B. Betz

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Publication 2

Gene-activated tissue grafts for sustained bone morphogenetic protein-2 delivery and bone engineering: Is muscle with fascia superior to muscle and fat?

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Abbreviations

MSC	=	Mesenchymal stem cell
MN	=	normal growth medium
MO	=	osteogenic medium
GFP	=	green fluorescence protein
Ad.GFP	=	adenoviral vector carrying the green fluorescent protein gene
BMP	=	bone morphogenetic protein
Ad.BMP-2	=	adenoviral vector carrying the bone morphogenetic protein gene
pfu	=	plaque forming units
DMEM	=	Dulbecco´s modified eagles medium
PBS	=	phosphate buffered saline
WST-1	=	water-soluble tetrazolium-1
CMV	=	cytomegalovirus
BAC	=	bacterial artificial chromosome
HEK	=	human embryonic kidney
ELISA	=	enzyme-linked immunosorbent assay
ALP	=	alkaline phosphatase
OCN	=	osteocalcin
OPN	=	osteopontin
Scl	=	Sclerostin
GAPDH	=	gadolinium phosphate dehydrogenase

Introduction

Bone fractures and bone defects occur in a variety of accidents, including traffic accidents, slip and fall accidents and other traumatic accidents. Clinical treatment with surgical or conservative therapies aims at obtaining bone healing with maximum functional recovery. However, delayed unions and non-unions are still presented the common complications, especially among long bone fractures. Up to 5%-10% of bone fractures will develop a non-union (Kostenuik & Mirza, 2017; Panteli, Pountos, Jones, & Giannoudis, 2015), depending on the location and severity of the bone injury, soft tissue and vascular damage, the treatment for which remains great clinical challenges around the world.

Autologous bone grafts have been considered as "gold standard" for large critical bone defects and non-unions because of their osteoinductive and osteoconductive properties (Heiple, Chase, & Herndon, 1963; Rogers & Greene, 2012; Ryzewicz et al., 2009). However, the limited supply and associated donor site morbidity constrain its further clinical application. Despite various research and methods have been investigated to develop new effective treatment strategies that can replace the current gold standard, little progress having been made.

Mesenchymal stem cells (MSCs) have gained steady attention in the field of tissue engineering and regenerative medicine due to their multi-directional differentiation potential. Under specific differentiation conditions, MSCs are capable to differentiate into bone, cartilage, as well as muscle, fat and other soft tissues. In many cell-based tissue engineering studies for bone repair, MSCs in combination with various novel scaffolds have shown their promising achievements (del Rosario, Rodríguez-Évora, Reyes, Delgado, & Évora, 2015; J. J. Li, Roohani‐Esfahani, Kim, Kaplan, & Zreiqat,

2017; Niu et al., 2015; Schütz et al., 2017). However, cell isolation and time-consuming expansion procedures prior implantation obviously increase the cost and may induce the risk of contamination (Aslan et al., 2006). In addition, the cell-based tissue engineering approach has very high demands for scaffolds in terms of material and property to provide a suitable microenvironment for cell migration, proliferation and differentiation (Tolar et al., 2007). The complexity of such cell-based approaches challenges their practical translation.

The improved strategy is utilizing tissue grafts instead of stem cells, so that the procedure of cell isolation and propagation could be avoided. This clearly simplifies the previous cell-based therapies and therefore are better suited for a clinical application. As indicated by Betz et al. (O. B. Betz et al., 2009; V. M. Betz et al., 2016), large segmental bone defects could be healed by the implantation of bone morphogenetic protein (BMP) gene activated fat tissue grafts. Despite fat tissue is recognized as the tissue source of adipose derived stem cells (ADSCs), whether gene activated fat tissue implant can transdifferentiate into bone is still unknown, while it could also be explained by that the gene transduced tissue implant merely serves as molecule signals delivery system, which mobilize host stem cells or progenitor cells to the injury site and then activate the body's own regenerative capacity.

In the publication 1, our study was designed to investigate the osteogenic transdifferentiation potential of human fat tissue under an osteogenic stimulus *in vitro*. Additionally, the capacity of three recombinant growth factors BMP -2, BMP-7 and BMP-9 to induce osteogenic transdifferentiation of adipose tissue was explored and compared.

The result demonstrated for the first time that human fat tissue is capable to undergo osteogenic transdifferentiation directly *in vitro*, and recombinant human BMP-9 was identified to be the most potent osteogenic inducer (Bondarava et al., 2017). Inspired by this, our following work focused on exploring growth factor delivery system to optimize osteogenic transdifferentiation for this novel tissue-based treatment.

BMPs with osteogenic activity has attracted extensive interest as growth factors for enhanced bone construction. In particular, BMP-2, with the most effective osteogenic activity among BMPs, has been extensively used in non-union bone fractures, segmental bone defects and spinal fusions (O. B. Betz et al., 2010; Mumcuoglu et al., 2018), since the Food and Drug Administration (FDA) approved their clinical use for specific indications in 2002 (McKay, Peckham, & Badura, 2007).

BMPs are considered to be promising stimulators for bone reconstruction, but their stimulation ability is still largely dependent upon the delivery vehicle. It has been reported that at an equivalent dose of BMP-2, sustained delivery exhibited enhanced osteogenesis as compared to short-time delivery (Jeon et al., 2008; Yang et al., 2010). The short biological half-life of recombinant BMP proteins and rapid loss through diffusion necessitate an improved delivery system that can regulate sustained release of BMP-2 (Jung et al., 2017). Applying large amounts of recombinant hBMP-2 may compensate for the fast degradation of protein but more often leads to unexpected side effects (Hajimiri, Shahverdi, Kamalinia, & Dinarvand, 2015).

In order to achieve sustained delivery of BMPs and thereby a long-lasting bone regeneration stimulus, gene transfer technologies have been developed. It has been revealed that gene transduction with BMPs can significantly accelerate osteogenic differentiation and mineralization of stem cells in monolayer (Zachos, Shields, &

Bertone, 2010) or three-dimensional culture (Hao et al., 2010; Meinel et al., 2006). Meinel et al. compared the osteogenic performance of adenovirus encoding BMP-2 (Ad.BMP-2) transduced MSCs and non-transduced MSCs but exposed to the same time concentration profile of BMP-2 generated by supplementing growth factor to culture medium, demonstrating that gene transduction resulted in higher levels of expression of osteogenic marker genes (Meinel et al., 2006). However, the disadvantage of such cell-based gene enhanced tissue engineering approaches is that, as mentioned before, it requires an additional step of cell isolation and cultivation and therefore increasing time and cost (Aslan et al., 2006). Our strategy is to utilize gene activated soft tissue fragments as regenerative implants, which integrate stem cells, growth factors and scaffolds, the three key components of tissue engineering.

Betz et al. have utilized muscle and subcutaneous fat tissue transduced with adenoviral BMP-2 vector to repair large bone defects in rats (Betz et al., 2009; Betz et al., 2010). The tissue grafts were brought in contact directly with the gene transferring vectors through transduction, leading to the genetically modification of cells on the surface of the tissue fragments. The so called "gene activated tissue grafts" represent biological regenerative implants providing growth factors and stem cells to the injury site.

Subcutaneous fat tissue has led to a new focus towards bone regeneration as an abundant source of adipose derived stem cells. Numerous studies have shown the multi-lineage differentiation capacity of ADSCs (Awad, Wickham, Leddy, Gimble, & Guilak, 2004; Luzi et al., 2008; Zuk et al., 2002). More recently, the investigation into whether differentiation could be induced in the fat tissue fragments is drawing increasing attention. One *In vitro* study has revealed that under osteogenic stimulus, human fat tissue is capable to transdifferentiate into bone-like tissue (Bondarava et al., 2017).

Another *in vivo* study applied Ad.BMP-2 activated fat tissue in order to heal critical-size bone defects in rats (O. B. Betz et al., 2010).

Skeletal muscle tissue represents another convenient source of stem cells, and thus holds great promise to serve as therapeutic autografts for bone regeneration. Various studies have applied muscle derived stem cells to repair or regenerate muscular disease, bone and cartilage defects as well as nerve injury (Lavasani et al., 2014; Matsumoto et al., 2009; Ota et al., 2011; Wright et al., 2002). In an expedited *ex vivo* gene therapy approach, muscle tissue fragments could be directly activated by gene transduction without prior cell isolation, leading to a rapid healing of large segmental bone defects (O. B. Betz et al., 2009).

The fascia of skeletal muscle is a sheet of connective tissue consisting of fibroblasts. Although a growing number of studies have demonstrated that multipotent progenitor cells reside in fascia, few studies were performed investigating its regenerative capacity. It was recently reported that fascia derived stem cells possess chondrogenic potential (G. Li, Zheng, Meszaros, Vella, & Corsi, 2011). Wong et al. confirmed the multi-lineage differentiation potential of fascia, including chondrogenic differentiation, osteogenic differentiation and adipogenic differentiation (Wong et al., 2015). As part of my thesis, I tried to answer the question whether the large number of fibroblasts in fascia could be effectively transduced by adenoviral vectors, making it a potential vehicle for growth factors delivery and thus enhancing osteoinduction of muscle tissue.

Although gene activated fat tissue and muscle tissue's promising capacity as regenerative implants for bone repair have been explored by several *in vivo* studies, to the best of our knowledge, few studies have investigated the hBMP-2 protein expression profile of genetically modified tissue fragments. It was also still unknown if

gene transduction or osteogenic differentiation of muscle tissue could be promoted by a fascia layer.

Hence, the goal of the second publication was to evaluate and compare the potential of fat, muscle alone, and muscle with fascia tissue fragments to serve as BMP-2 delivering vehicles. In addition, the applied dosage of adenovirus BMP-2 vectors for transduction was optimized in order to achieve an ideal production of growth factor for future bone regeneration applications.

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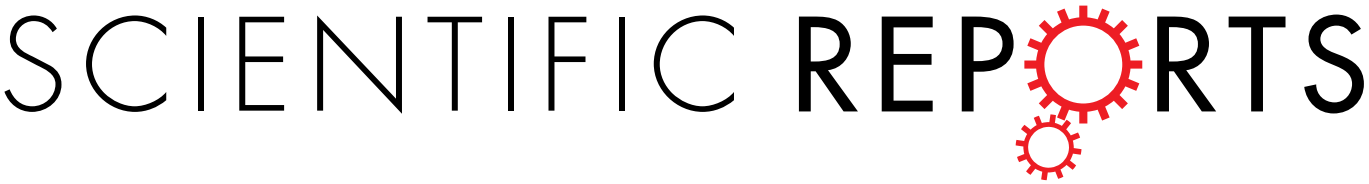
Publication 1



OPEN

Osseous differentiation of human fat tissue grafts: From tissue engineering to tissue differentiation

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Conventional bone tissue engineering approaches require isolation and *in vitro* propagation of autologous cells, followed by seeding on a variety of scaffolds. Those protracted procedures impede the clinical applications. Here we report the transdifferentiation of human fat tissue fragments retrieved from subcutaneous fat into tissue with bone characteristics *in vitro* without prior cell isolation and propagation. 3D collagen-I cultures of human fat tissue were cultivated either in growth medium or in osteogenic medium (OM) with or without addition of Bone Morphogenetic Proteins (BMPs) BMP-2, BMP-7 or BMP-9. Ca^{2+} depositions were observed after two weeks of osteogenic induction which visibly increased when either type of BMP was added. mRNA levels of alkaline phosphatase (ALP) and osteocalcin (OCN) increased when cultured in OM alone but addition of BMP-2, BMP-7 or BMP-9 caused significantly higher expression levels of ALP and OCN. Immunofluorescent staining for OCN, osteopontin and sclerostin supported the observed real-time-PCR data. BMP-9 was the most effective osteogenic inducer in this system. Our findings reveal that tissue regeneration can be remarkably simplified by omitting prior cell isolation and propagation, therefore removing significant obstacles on the way to clinical applications of much needed regeneration treatments.

A growing aging population with an increased risk of bone fractures due to falls[1], unfortunately often combined with impaired bone healing and even higher fracture risk due to osteoporosis[2] and diabetes[3,4], and, in addition, to the currently inevitable loosening of prosthetic implants over time[5], urgently requires adequate bone regeneration strategies. The situation is exacerbated by a rising number of devastating traumatic war injuries for which often no other treatment option than amputation exists due to the extensive loss of bone and soft tissue[6]. Current treatment options are associated with high morbidity[7–9] or deficient efficacy[10]. Mesenchymal stem cells (MSCs) in combination with various scaffolds are under intensive investigation and show promising achievements[11–14]. However, the involved procedure of cell isolation or separation is costly, leading to an estimated market worth 6.3 billion USD by 2020[15]. Current tissue engineering strategies also involve subsequent *in vitro* propagation of the prior isolated or separated cells. These procedures add further substantial costs which is reflected in an estimated market worth 14.8 billion USD by 2019[16]. Extended cultivation time also holds a concerning higher risk of contamination and unwanted effects due to prolonged exposure to the cell culture media[17]. The cell propagation is currently done in external GMP-Facilities, which again adds costs, time and risks to the cell product. To avoid the issues afflicted with the use of an external GMP-Facility, the idea of a "GMP in a box", in form of a fully automated bench-top culture system within the primary-care facility of the patient seems very promising[18]. Not only would the use of tissue grafts instead of isolated and propagated cells clearly simplify such a system, therefore accelerating the availability, but because even when performed in the operating room and used autologously, cell separation is considered "more than minimally manipulated" by the FDA[15], requiring a more rigorous approval process. Tissue grafts could therefore remove another obstacle on the road to a clinical application. Lastly, fat tissue grafts have

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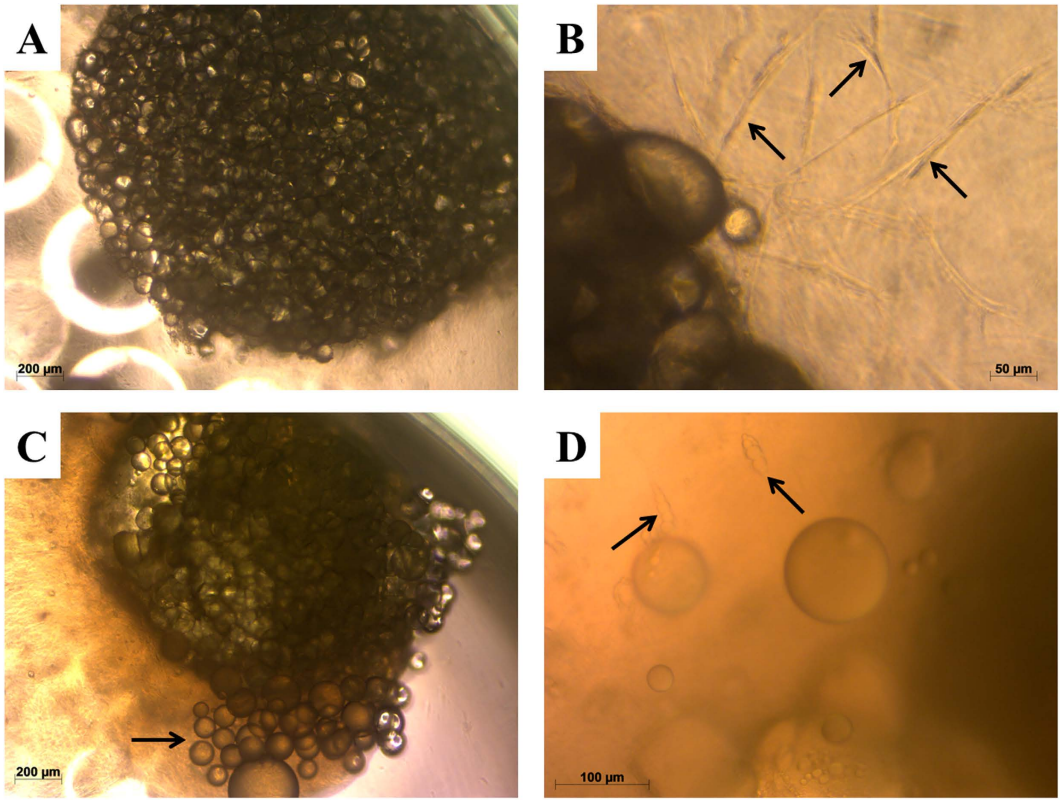

Figure 1. Phase contrast images of collagen-I fat cultures. (**A**) Freshly prepared culture. (**B**) Migration of spindle-shaped cells (arrows) out of the fat tissue in the collagen-I gel after 1 week of culture. (**C**) Migration of adipocytes (arrow) out of the fat tissue after 2 weeks of culture. (**D**) Spindle-shaped cells containing fat droplets (arrows) migrating into the collagen-I gel after 3 weeks of culture.

the potential to further the demand by surgeons for a same day, *ex-vivo* therapy[19]. For all those reasons above, it becomes obvious that technologies without the requirement of cell isolation and propagation would increase the chances to meet the need of the increasing number of patients for bone regeneration. Preclinical studies, showing that implantation of fat or muscle tissue fragments transduced with an adenoviral BMP-2 vector induces structural and functional healing of large segmental bone defects, were recently reported[20–23]. However, it remained unclear, whether the BMP-2 transduced tissue graft itself can undergo transdifferentiation into bone or if the graft rather serves as a delivery system for growth factors which stimulate and attract stem cells of the surrounding tissue[21]. If the latter would be the case, it would be advised to focus rather on drug delivery optimisation than cell therapies. Therefore, the aim of the present study was to investigate whether human fat tissue containing inhomogeneous cell populations is capable of transdifferentiation into tissue with bone characteristics. Adipose tissue harvest is associated with minimal donor site morbidity and it represents an especially appealing source of progenitor cells that can be used for the repair of bone[21,24]. A new culture system, introduced by Sonoda *et al.*[25], represents adipose tissue fragments embedded in a three-dimensional collagen gel. This culture method was preferred in the present study not only because of the ability to easily entrap buoyant adipose tissue fragments, but also due to the support of a three-dimensional tissue structure mimicking an *in vivo* situation more closely and providing favourable conditions for cell differentiation. Bone morphogenetic proteins (BMPs) are known as bone forming growth factors. BMP-2 and BMP-7 have been shown to induce osteogenesis *in vivo*[26–34], and both are available for clinical use[35–37]. Another promising growth factor inducing formation of bone is BMP-9. The superior potential of BMP-9 to induce osteogenic differentiation of MSCs was demonstrated both *in vivo* and *in vitro*[38–46]. However, no studies comparing the influence of BMP-2, BMP-7 and BMP-9 on human fat tissue fragments are available so far.

In this study, we used a modified, three-dimensional collagen I gel system to culture human subcutaneous fat tissue fragments *in vitro*. We evaluated whether cultivation with osteogenic supplements leads to tissue calcification and expression of osteoblast specific protein markers. Additionally, the potential of three recombinant growth factors BMP-2, BMP-7 and BMP-9 to induce osteogenic transdifferentiation of fat tissue was explored and compared.

Results

3-Dimentional fat tissue culture system. After being embedded in collagen I gel, adipose tissue fragments appeared under the phase contrast microscope as mature adipocytes attached to each other (Fig. 1A). After 5–7 days in culture, spindle-shaped cells were observed at the peripheral zones of the fragments migrating

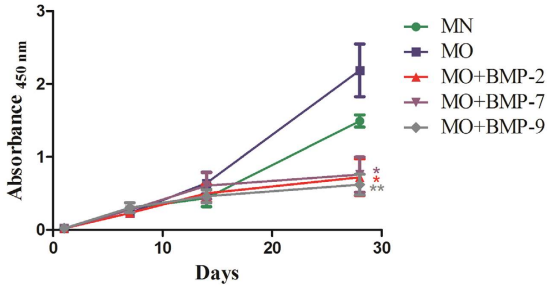

Figure 2. WST-1 measurement of fat tissue cultures under different differentiation culture conditions. Values given represent means ± SE, n = 6–7. The level of significance was set as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

into the collagen I gel (Fig. 1B). Furthermore, after the second week of culture, some adipocytes started to dissociate from the tissue conglomerate. This could be observed exceedingly in samples cultured in normal growth medium (MN) (Fig. 1C). In addition to adipocytes and spindle-shaped cells, some preadipocyte-like cells with small fat droplets were observed in the late stages of the culture period of 4 weeks (Fig. 1D). Collagen-I gel was continuously shrinking during the whole period of incubation and losing its transparency under the osteogenic conditions. The migrating cells were spread throughout the collagen gel, partly covering the bottom surface of the 96-well plates.

Cell viability and proliferation. Cell viability and proliferation in the fat fragments was not only maintained for four weeks, but increased progressively over time in all experimental groups (Fig. 2). The highest WST-1 absorbance values, as an indicator of cell viability and proliferation, were observed in the osteogenic medium (MO) group: 0.23 ± 0.037 after 1 week, 0.64 ± 0.15 after 2 weeks, and 2.19 ± 0.36 after 4 weeks of incubation. Less increase in absorbance was observed in cultures treated with normal media (MN): 0.29 ± 0.06 after 1 week, 0.44 ± 0.12 after 2 weeks, and 1.49 ± 0.08 after 4 weeks of incubation. However, no significant difference was detected between the MO and MN groups. In contrast, significant lower absorbance values in comparison to MN were detected in all groups treated with BMPs after 4 weeks of incubation: 0.23 ± 0.04; 0.50 ± 0.12; 0.72 ± 0.25 ($p \leq 0.05$) after 1, 2 and 4 weeks respectively in the MO-BMP-2 group; 0.27 ± 0.04; 0.61 ± 0.18; 0.76 ± 0.24 ($p \leq 0.05$) after 1, 2 and 4 weeks respectively in the MO-BMP-7 group; 0.30 ± 0.07; 0.46 ± 0.09; 0.62 ± 0.14 ($p \leq 0.01$) after 1, 2 and 4 weeks respectively in the MO-BMP-9 group, indicating a significant lower proliferation rate in the BMP receiving groups. No significant difference between the BMP-groups was observed.

Histology. After one week of incubation, none of the five groups displayed calcium depositions. (Figs 3A,D,G,J,M and 4A,D,G,J,M). After the second week of incubation, the samples treated with MN showed a higher number of cell nuclei located between the adipocytes than compared to cultures under osteogenic conditions. This suggests a higher proliferation of stromal cells in the MN group. Samples treated with MO displayed some regions of calcification located in the stromal cell fraction between adipocytes. The fat grafts of the BMP-2, -7 and -9 groups displayed a remarkable increase in extracellular calcification, as confirmed by alizarin red S as well as by von Kossa staining (Figs 3H,I,K,L,N,O and 4H,I,K,L,N,O). After the fourth week of incubation the proliferation of the stromal cells in the MN group increased even further replacing adipocytes almost completely. The samples treated with MO and MO + BMPs demonstrated a strong overall tissue calcification, which included the adjacent collagen I gel. There was no significant difference in the calcification rate observed between MO and MO + BMP-2, -7 or -9 experimental groups after the 4th week of incubation.

Real-time quantitative PCR (RT-PCR). Osteogenic differentiation of the 3D cultures was confirmed by quantitative real-time PCR measuring the expression of the early bone marker ALP and the later bone marker OCN mRNA. The expression of these markers in native fat tissue of each patient served as a baseline (calibrator). The mRNA expression of the early bone marker ALP after one week of culture was significantly ($p \leq 0.001$) higher in samples treated with MO plus BMP-2 (2.87 ± 0.46), BMP-7 (2.78 ± 0.56) and BMP-9 (6.90 ± 2.00) compared to samples treated with MN (0.66 ± 0.15) or MO (0.80 ± 0.19, $p \leq 0.01$ or $p \leq 0.001$). The ALP-expression for MO plus BMP-9 was significantly higher than for all other groups (Fig. 5a). After the second week of culture the level of ALP in the MO + BMP-9 group was still significantly higher (4.75 ± 0.82, $p \leq 0.01$) than in the other groups: MN (0.81 ± 0.27), MO (1.08 ± 0.28), MO + BMP-2 (1.56 ± 0.31), MO + BMP-7 (0.55 ± 0.07). However, after the fourth week of incubation the highest level of ALP expression was observed in the MO group (2.16 ± 0.39, $p \leq 0.001$). The groups MO + BMP-2, MO + BMP-7 and MO + BMP-9 showed significantly higher ALP expression values (0.84 ± 0.12; 1.35 ± 0.31; 1.64 ± 0.32 respectively, $p \leq 0.05$ or $p \leq 0.001$) compared to the MN group (0.48 ± 0.14) as well (Fig. 5a).

OCN mRNA expression after the first week of culture was significantly higher in the samples treated with MO containing BMP-2 (0.30 ± 0.05), BMP-7 (0.40 ± 0.09) or BMP-9 (0.93 ± 0.29) than in samples treated with MN (0.14 ± 0.03, $p \leq 0.05$) or MO (0.12 ± 0.02, $p \leq 0.01$ or $p \leq 0.001$) (Fig. 5b). After the second week of culture the level of OCN expression was significantly higher in the samples treated with MO (0.21 ± 0.03, $p \leq 0.05$), MO + BMP-2 (0.43 ± 0.10, $p \leq 0.001$), MO + BMP-7 (0.30 ± 0.06, $p \leq 0.01$) and MO + BMP-9 (0.29 ± 0.04, $p \leq 0.001$) compared to the samples treated with MN (0.11 ± 0.02). After the fourth week of incubation the

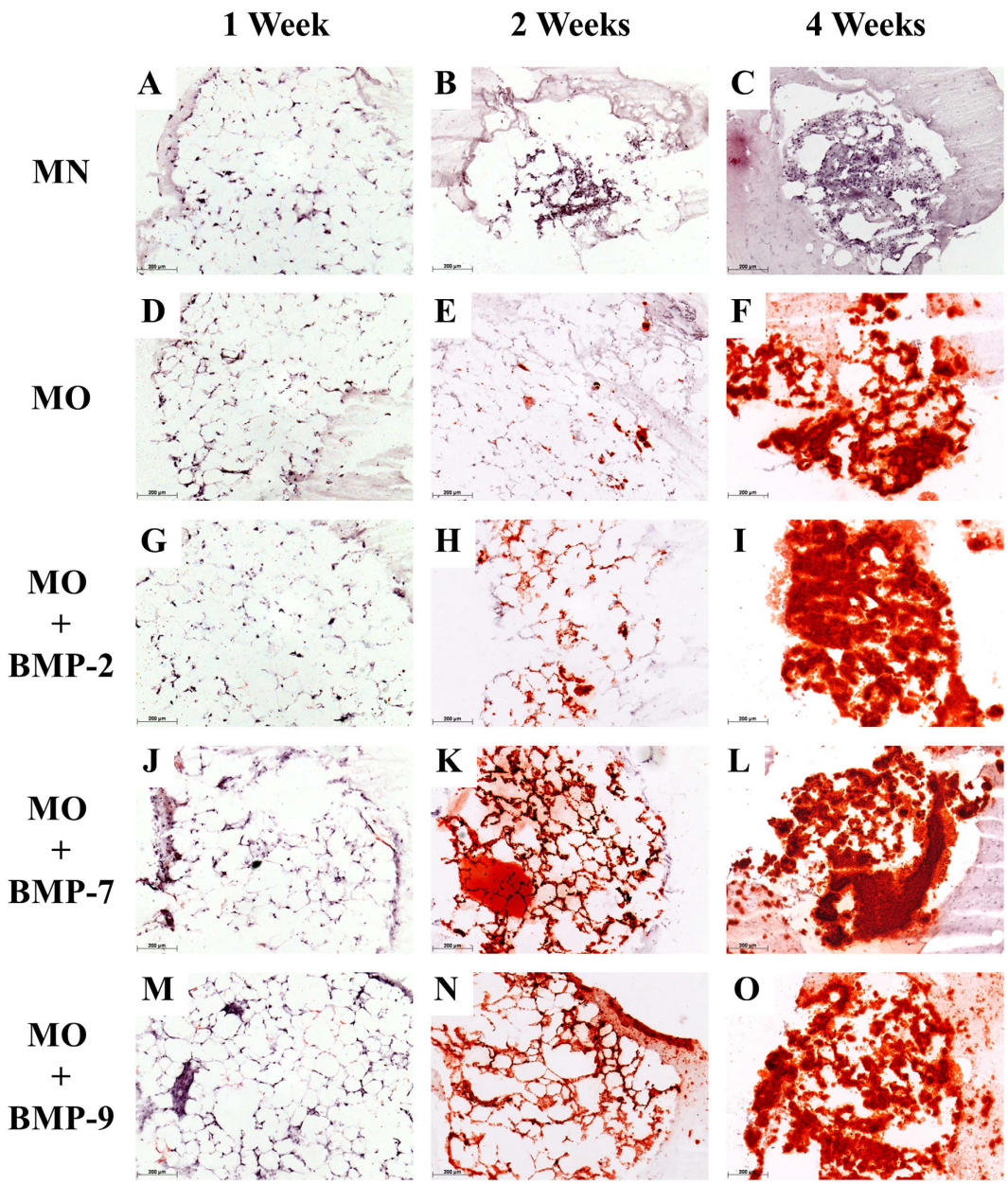

Figure 3. Histological sections of fat cultures after 1, 2 or 4 weeks under various differentiation conditions. Sections were stained with Alizarin red S: 1 week: (**A**) MN, (**D**) MO, (**G**) MO + BMP-2, (**J**) MO + BMP-7, (**M**) MO + BMP-9; 2 weeks: (**B**) MN, (**E**) MO, (**H**) MO + BMP-2, (**K**) MO + BMP-7, (**N**) MO + BMP-9; and 4 weeks of incubation: (**C**) MN, (**F**) MO, (**I**) MO + BMP-2, (**L**) MO + BMP-7, (**O**) MO + BMP-9. Scale bar = 200 μm.

OCN mRNA was significantly overexpressed in the groups MO, MO + BMP-2, MO + BMP-7 and MO + BMP-9 (0.60 ± 0.03, $p \leq 0.001$; 0.56 ± 0.13, $p \leq 0.001$; 0.47 ± 0.12, $p \leq 0.01$ and 0.90 ± 0.28, $p \leq 0.001$ respectively) compared to to the MN group (0.14 ± 0.03) (Fig. 5b).

Immunofluorescence. Immunofluorescent staining of native fat tissue fragments for the bone markers osteocalcin (OCN) and osteopontin (OPN) showed that these proteins were endogenously expressed at very low levels in the adjacent blood vessels and in some niches of the stromal cells within fat tissue, but not in the adipocytes (Fig. 6A,B). In contrast, sclerostin could not be detected in native fat samples (Fig. 6C). After 4 weeks of culture in MN, the bone markers OCN and OPN could still be detected intracellular, although at minimal levels, mostly localized in the cell nuclei (Fig. 6D,E). Expression of sclerostin was not observed in MN cultures (Fig. 6C,F). Samples treated with MO displayed a low fluorescence signal for OCN and OPN (Fig. 6G,H) and a positive signal for sclerostin (Fig. 6I). All the samples treated with MO and supplemented with BMPs (BMP-2, BMP-7, BMP-9) showed strong fluorescence signals for OCN, OPN and sclerostin (Fig. 6J–R). There was no apparent difference observed concerning the expression of OCN and OPN between the three different BMPs. Sclerostin expression appeared to be higher in samples treated with MO + BMP-7 and MO + BMP-9 compared

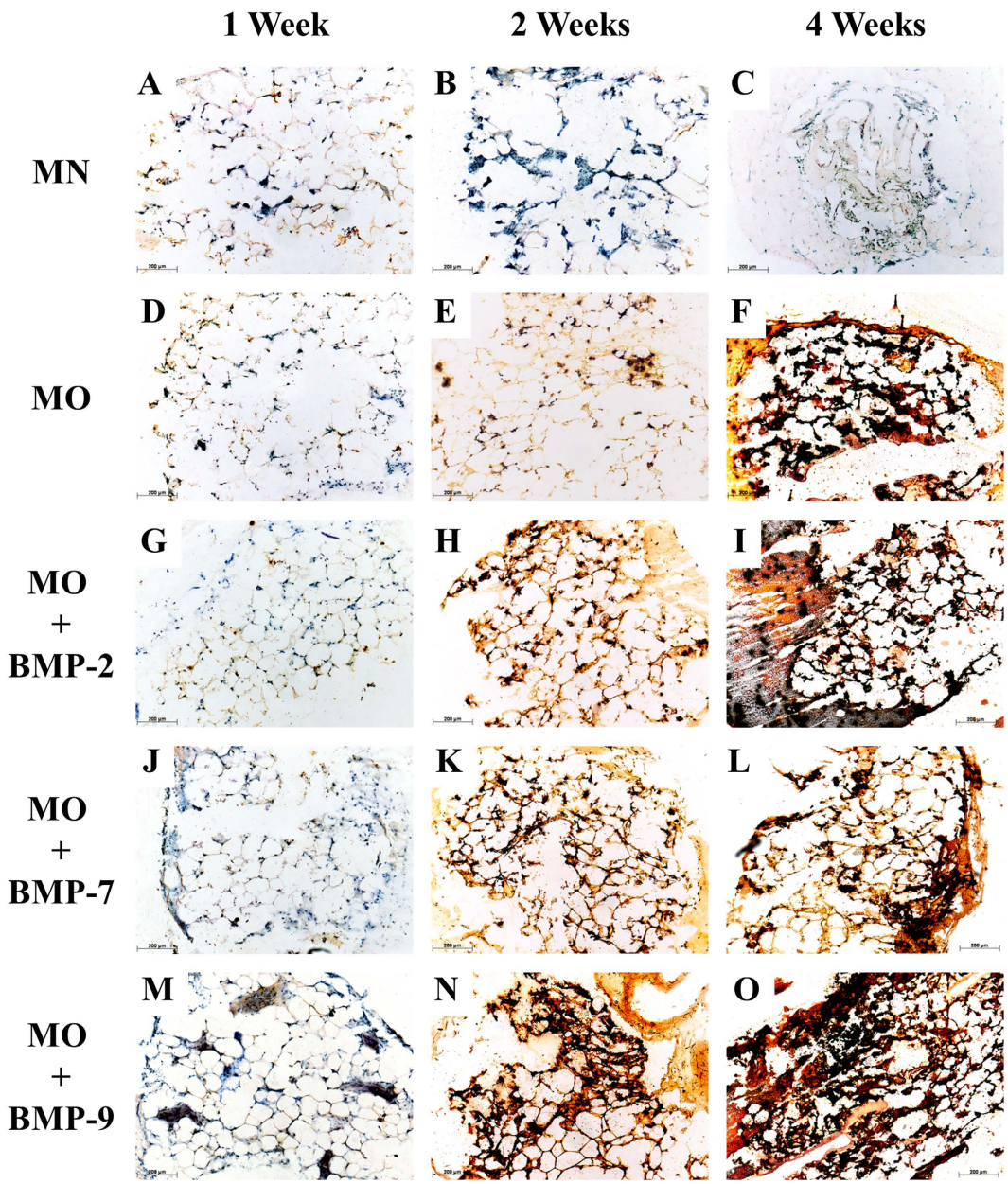

Figure 4. Histological sections of fat cultures after 1, 2 or 4 weeks under various differentiation conditions. Sections were stained with von Kossa: 1 week: (**A**) MN, (**D**) MO, (**G**) MO + BMP-2, (**J**) MO + BMP-7, (**M**) MO + BMP-9; 2 weeks: (**B**) MN, (**E**) MO, (**H**) MO + BMP-2, (**K**) MO + BMP-7, (**N**) MO + BMP-9; and 4 weeks of incubation: (**C**) MN, (**F**) MO, (**I**) MO + BMP-2, (**L**) MO + BMP-7, (**O**) MO + BMP-9. Scale bar = 200 μm.

to MO + BMP-2 (Fig. 6L,O,R). Furthermore, fluorescence signals from OCN, OPN and sclerostin in samples treated with MO + BMPs were observed not only intracellular, but also extracellular and to some extend in the surrounding collagen I matrix.

Discussion

In this study we employed a 3D fat tissue culture system established by Sonoda *et al.*[25] with minor adaptations. Despite our adaptations, most of the vitality and histological data were in line with the results found by Sonoda *et al.*[25]. The fat tissue cultures in collagen I gel did not only retain their viability but rather displayed increasing vitality parameters over 4 weeks. The migration and proliferation of spindle-shaped cells were observed at the periphery of the fat tissue fragments. Cells of the same phenotype were shown by Sonoda *et al.* (Fig. 1B) to express CD 44 and CD 105 – the surface markers of mesenchymal stem cells[25]. Capable of migration and proliferation, these cells might contribute to the osteogenic differentiation described in the present study. The dissociation of adipocytes at the late phase of the tissue culture was not shown before[25]. A possible reason could be the human origin of the tissue as opposed to the rat origin used by Sonoda and the difference in culture conditions. Only very few spindle-shaped cells containing lipid droplets (Fig. 1D) were observed in the cultures, making it

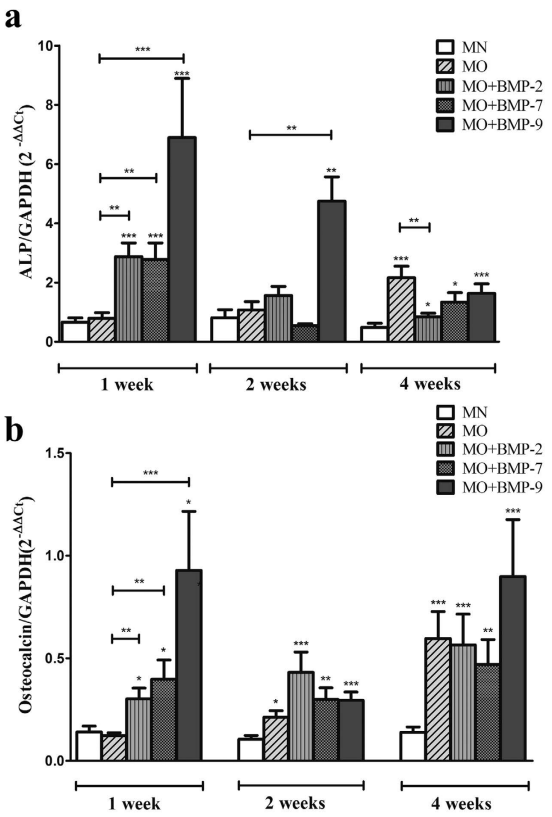

Figure 5. Quantitative RT-PCR analysis of 3D fat cultures incubated under different differentiation conditions. Expression of mRNA for ALP (**a**) and OCN (**b**) normalized to mRNA levels for GAPDH. Values given represent means ± SE, n = 10–11. The level of significance was set as * for $p < 0.05$, ** for $p < 0.01$ and ***for $p < 0.001$.

highly unlikely, that these cells are exclusively responsible for the osteogenic differentiation of the fat grafts. This observation rather suggests the presence of preadipocytes or dedifferentiated adipocytes within the fat tissue. Moreover, the vitality of the 3D cultures was continuously increasing during the first two weeks of culture with no significant difference between the experimental groups (Fig. 2). However, after 4 weeks of incubation the vitality of groups containing recombinant BMPs (BMP-2, -7 and -9) was significantly lower than in MN and MO groups. Addition of osteogenic growth factors to the cultures caused an accelerated differentiation rate but also a decrease of the proliferation rate. This was to be expected as cell differentiation has long been recognised as an opposing process to cell proliferation[47].

Accumulation of calcium under osteogenic conditions was observed mostly among mature adipocytes at the sites of stromal cell localisation, or in the niches of progenitor cells (Figs 3 and 4). Presumably, only these cells produce a sufficient number of progenitors that can respond to osteogenic stimuli and differentiate into the respective lineage. Moreover, calcification in samples containing BMPs was observed in the collagen I gel adjacent to the fat tissues, suggesting the contribution of migrated cells to the overall calcification (Figs 3L,O and 4I).

Calcium accumulation during osteogenic differentiation was already described in several studies on adipose-derived stem cells cultured in monolayer[24,48] or in 3D cultures[49,50]. To the best of our knowledge, this reported study demonstrates for the first time, osteogenic differentiation, including calcification of 3D fat tissue cultures of human origin *in vitro*. Although, Betz *et al.*[20] have demonstrated that BMP-2 gene-activated fat tissue is an osteoregenerative material that has the ability to repair critical-sized bone defects in rats[21], it was still unclear, whether the gene-activated tissues expressing BMP-2 transform into bone or solely serve as a delivery system for BMP-2 stimulating and attracting stem cells of the surrounding tissue. The presented data substantiates our hypothesis that fat tissue has the potential for the transition into bone tissue.

The RT-PCR data reveals increased mRNA expression of the bone markers ALP and OCN under osteogenic culture conditions. Moreover, the addition of BMP-2, -7 and -9 seems to induce osteogenesis in the fat tissue earlier and more intensively than osteogenic medium alone. These data were supported by the immunofluorescent staining for OCN, OPN and Scl. Furthermore, Scl appears to be the most specific osteogenic marker in this system, as it was only detectable after osteogenic stimulation but not in the native or in MN cultured tissue. The presented data support the hypothesis, that osteogenesis can be induced in human fat tissue without isolation of progenitor cells. The presence of mesenchymal stem cells and their osteogenic potential were confirmed for different types of adipose tissue by numerous studies[48,50,51]. However, no data was reported so far on fat tissue transdifferentiation *in vitro*. Limited *in vivo* data reporting transdifferentiation between white and brown types of fat cells[52] as well as fat-epithelial cell differentiation[53,54] was reported. Moreover, Gao *et al.*[55] described recent

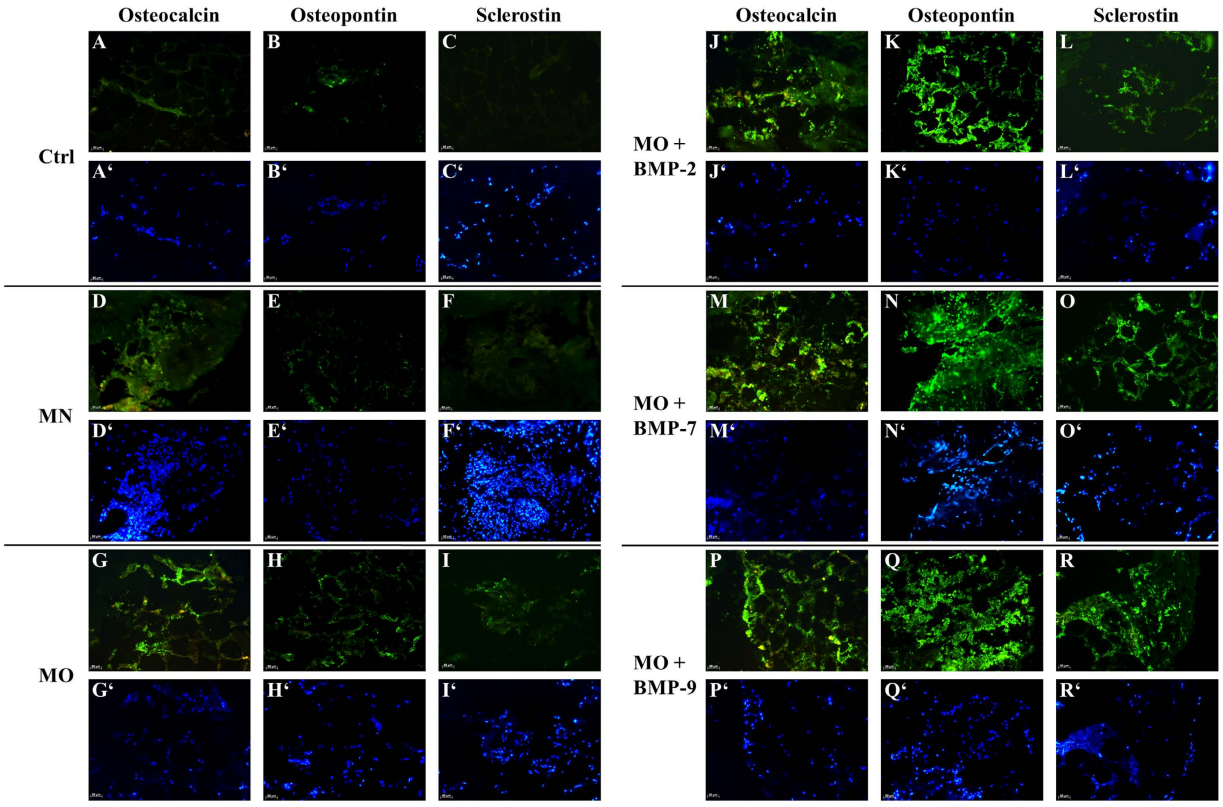

Figure 6. Histological sections of fat cultures after 4 weeks under various differentiation conditions. Immunofluorescence for OCN, OPN and Scl is shown in green. Control (Ctrl) samples (**A**–**C**) represent the untreated fat fragments. Samples treated with MN are shown in the images (**D**–**F**); MO at (**G**–**I**); MO + BMP-2 in (**J**–**L**); MO + BMP-7 in (**M**–O); MO + BMP-9 in (**P**–**R**). Nuclei of cells (blue) are shown in the corresponding images (**A'**–**R'**). Scale bar = 50 μm.

insights in studying transdifferentiation of bone and fat related to bone metabolism. Subsequent research revealed the capacity of subcutaneous preadipocytes to differentiate into osteoblasts[56]. Other studies reported, that mature lipid-filled adipocytes transdifferentiated into mature osteoblasts through the preadipocytic stage with increased cell proliferation[57,58]. Also, it was discovered that single adipocytes are capable to dedifferentiate into cells with fibroblast-like morphology and subsequently turn into osteoblasts or adipocytes under respective stimulation[58,59].

The osteogenic BMPs hold great promise for regenerative medicine[60]. Our results have demonstrated that BMP-2, -7 and -9 represent osteogenic activities and enhance osteogenic differentiation of human fat tissue *in vitro* at the mRNA and protein level. Moreover, BMP-9 seems to be a more potent osteogenic inducer for cells within fat tissue than BMP-2 or -7. This observation is in line with the previous studies conducting comprehensive analysis of different types of human BMPs[39,41,61,62]. By using recombinant adenoviruses in order to facilitate the expression of 14 different BMPs, Luu *et al.*, 2006 have demonstrated that, besides BMP-2 and BMP-7, BMP-6 and BMP-9 show the highest osteogenic activity *in vitro* as well as *in vivo*[39]. Li *et al.*, 2003 have reported that BMP-9 displays the greatest osteogenic potential *in vitro* and in Sprague-Dawley rats[62]. Furthermore, Kang *et al.*[41] suggested that high osteogenic activity of BMP-9 may be explained by transduction of a distinct osteogenic signalling pathway that is completely different from that of BMP-2, BMP-6 and BMP-7[41]. One reason for the osteogenic potency of BMP-9 is most likely the fact, that it cannot be inhibited by noggin, an effective inhibitor of BMP-2 and -7[63,64]. This would also explain why BMP-9 is involved in traumatic, heterotopic ossification where small amounts of BMP-9 can induce ossification of soft tissue in humans[65].

In conclusion, we have demonstrated for the first time that human fat tissue is able to undergo osteogenic transdifferentiation under 3D culture conditions *in vitro,* although it remains unclear which compartments of the heterogenic cell population within the fat tissue are responsible for the differentiation process. We furthermore evaluated the osteogenic potential of human recombinant BMP-2, -7 and -9 in this system. Here, BMP-9 was identified to be the most potent osteogenic inducer. Our results suggest that human subcutaneous fat tissue may represent a regenerative material, which could be applied without cell isolation in expedited bone regeneration strategies[21,23]. Future investigations should be directed toward understanding the cellular and molecular mechanisms of fat tissue transdifferentiation *in vitro* and *in vivo* and also toward the role of BMPs in this context with a potential focus on BMP-9.

At the clinical research front, the combinations of different BMPs and other growth factors should be evaluated for synergic osteogenic activity. In addition, alternative and safe BMP- delivery approaches need to be developed to enable efficient, safe and cost effective therapies for bone regenerative medicine.

Methods

Tissue Harvest and 3-Dimentional Culture System. The tissues and data used in this study were provided by the Biobank (http://www.klinikum.uni-muenchen.de/Chirurgische-Klinik-und-Poliklinik-Grosshadern/de/0800-gewebebank/index.html) located in the Hospital of the University of Munich, which operates in accordance with the European Union-compliant ethical and legal framework of the Human Tissue and Cell Research (HTCR) Foundation (http://www.htcr.org). The process of tissue collection included obtaining written informed consent from all tissue donors. The experimental procedures were performed within the framework of the HTCR. This framework has also been approved by the ethics commission of the Faculty of Medicine in the University of Munich and the Bavarian State Medical Association[66].

The subcutaneous fat tissue was obtained from 5 patients of different sex (age from 31–80, BMI 29–52). After rinsing with PBS containing 120 IU/ml penicillin/streptomycin and 0,375 μg/ml amphotericin B (Biochrom, Germany), the adipose tissue was punched with a biopsy punch to create fragments of approximately 2 mm in diameter. Each fragment was then embedded in 50 μl type I collagen gel solution (3D Collagen Culture Kit, Merck Millipore, Germany), which was prepared according to the manufacturer's protocol, and placed in a 96-well tissue culture plate. After polymerization of the collagen, the cultures were covered either with normal, maintaining media (MN) (Ham's F12/DMEM (Biochrom), 10% FCS (Sigma), 60 IU/ml penicillin/streptomycin) or osteogenic media (MO) (MN + 50 μM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, 10 nM dexamethasone) alone or with addition of one of the growth factors from the BMP superfamily (100 ng/ml): rhBMP-2, rhBMP-7 (Biomol, Germany) or rhBMP-9 (Bio-Rad, Germany). The media were changed every second day. Microscopic observations were performed using Axiovert 40 CFL equipped with AxioCam ERc 5 s (Carl Zeiss, Germany) after one day and after 1, 2 and 4 weeks of culture. Images were taken using AxioVision SE64 Rel. 4.9 software (Carl Zeiss, Germany). 6 cultures per one experimental group and time point were prepared and evaluated from each patient (90 cultures per patient).

Cell viability. Cell viability of the 3D fat cultures was determined using a water-soluble tetrasolium-1 (WST-1) reagent (Roche, Germany). The assay was performed after 24 hours and subsequently after 1, 2 and 4 weeks of culture. Briefly, the culture medium was discarded. The fresh growth medium was mixed with WST-1 at 10:1 (v/v), added to the wells with 3D cultures or to the empty wells (blanks) and incubated for 2 hours at 37 °C in 5% CO_2. After the incubation, 100 μl of medium-WST-1 mixture was transferred to a 96-well culture plate and the absorbance was read at 450 nm using Synergy HT microplate reader and Gen 5 2.03 software (BioTek, Germany). The assay was repeated 7 times in the independent cultures.

Histology. Fresh fat tissue samples or 3D fat cultures were embedded in Tissue-Tek O.C.T.™ Compound (Sakura, Germany) and frozen immediately in liquid nitrogen. Tissue sections of 14 μm were cut by the cryomicrotome (CM 3050, Leica, Germany), transferred onto Superfrost™ Plus microscope slides (Thermo Scientific, USA) and air-dried. All samples were prepared in triplets.

Alizarin red S staining. Deposition of calcium in the tissue was evaluated by means of Alizarin red S staining. The sections were fixed with 4% Paraformaldehyde (PFA) for 15 minutes at room temperature (RT) and rinsed with distilled water. They were then stained with freshly prepared 40 mM alizarin red S (Sigma) solution, pH 4,1 for 10 minutes at RT and washed in PBS. The samples were dehydrated in 100% Ethanol, cleared in Rothistol (Roth, Germany) and mounted with DPX (Merck).

Von Kossa staining. Slides fixed with PFA 4% and washed in distilled water were stained in 5% silver nitrate solution (Sigma) in the darkness for 5 minutes. They were then washed with distilled water and treated with 1% pyrogallic acid (Sigma) for 5 minutes. After washing and fixing with 5% NaOH for 4 minutes, the samples were stained with May-Grünwald stain (Sigma) for 10 minutes, dehydrated with 100% Ethanol, cleared with Rothistol and mounted with DPX.

RNA isolation and quantitative real-time PCR. The expression of ALP and OCN were analysed in combination with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after 1, 2 and 4 weeks of culture. Total mRNA was extracted using QIAzol Lysis Reagent (Qiagen, Gemany) according to manufacturer's protocol. Briefly, the cultures were frozen in liquid N_2 and homogenised using the Mikro-Dismembrator S (Sartorius Stedim Biotech, Germany). Subsequently, 1 ml QIAzol Lysis Reagent was added to the homogenate, resuspended and mixed with chloroform. After centrifugation, the upper aqueous phase was transferred to a new tube and mixed with isopropanol. The mRNA containing pellet was washed twice with 75% Ethanol, air-dried and redissolved in RNase-free water. After the incubation at 37 °C for 20 minutes RNA concentration and purity were assessed with the spectrophotometer (NanoDrop™ Lite, Thermo Scientific).

cDNA synthesis was performed for 15 minutes at 42 °C using Thermocycler (T100, Bio-Rad, Germany) in a reaction mixture containing 300 ng total RNA, buffers, Quantiscript reverse transcriptase and RT primer mix from QuantiTect Reverse Transcription-Kit (Qiagen, Germany) adjusted with RNase-free water to a total volume of 20 μl.

Amplification of the cDNA products was performed using the FastStart Essential DNA Green Master (Roche Applied Science, Germany) and the Light Cycler 96 (Roche, Germany). The reaction mixture contained 2,5 μl cDNA, 300 ng primer, 5 μl FastStart Essential DNA Green Master and RNase free water to a final volume of 10 μl. Primer sequences: GAPDH forward TGCACCACCAACTGCTTAGC, reverse GGCATGGACTGTGGTCATGAG (annealing temperature 60 °C); ALP forward TCAAGGGTCAGCTCC ACCACA, reverse ATTGGCCTTCACCCCACACA (60 °C); OCN forward CCCAGGCGCTACCTGTATCAA,

reverse CTGGAGAGGAGCAGAACTGG (64 °C). Primers were obtained from Metabion (Germany). Amplification was performed in triplicates in 96 well plates (Roche, Germany). Relative gene expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ method, considering GAPDH as a housekeeping gene and untreated fat tissue as a calibrator.

Immunofluorescent staining. The tissue sections were fixed with 4% PFA and washed in PBS with 0,1% Brij (Sigma). Non-specific binding sites were blocked by 5% bovine serum albumin (BSA) for 30 minutes. Thereafter, primary antibody was added and incubated overnight. All primary antibodies (OCN, OPN, Scl) were mouse monoclonal IgGs from R&D Systems (Germany) used in concentrations: 25 μg/ml (OCN, OPN) and 10 μg/ml Scl. For negative controls, the first antibody was omitted. After washing off unbound antibody and permeabilization with 0,1% Triton X-100 solution (Sigma) in PBS the secondary antibody goat antimouse IgG conjugated with Alexa Fluor 488 (Invitrogen, Germany) in the dilution 1:400 was added for 30 minutes, then intensively washed. Finally, the nuclei of cells were stained with Hoechst 33342 (Life Technologies, Germany). The slides were mounted with Fluoromount W (Serva Electrophoresis, Germany), air-dried and stored in darkness at 4 °C.

Microscopy was performed with the Zeiss Axioskop 40 equipped with appropriate filter sets and AxioCam MRc 5. Images were obtained with an Axio Vision, Rel. 4.9 software (Carl Zeiss, Germany).

Statistical data analysis. Data analysis was performed with Microsoft Excel 2010 and Prism 5.02 software for Windows. Values were presented as mean ± standard error. The Mann-Whitney U-test was used to determine differences between compared groups as a Gaussian distribution could not be assumed. The level of significance was indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

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Author Contributions

M. Bondarava and O.B. Betz: Substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data; participated in drafting the article for important intellectual content and gave final approval of the version to be submitted. C. Cattaneo and B. Ren: Substantial contributions to acquisition of data, analysis and interpretation of data; gave final approval of the version to be submitted. W.E. Thasler, V. Jansson and P. E. Müller Substantial contributions to conception and design, gave final approval of the version to be submitted.

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RESEARCH ARTICLE

Gene-activated tissue grafts for sustained bone morphogenetic protein-2 delivery and bone engineering: Is muscle with fascia superior to muscle and fat?

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Abstract

Previously, we have presented an expedited strategy for sustained delivery of bone morphogenetic protein-2 (BMP-2) to bone lesions based on the implantation of gene-activated fat and muscle fragments. The aim of the present in vitro experiments was to evaluate the potential of muscle with fascia as a BMP-2 delivering osteo-regenerative implant in comparison to fat tissue and muscle alone. Subcutaneous fat, muscle, and muscle with fascia were harvested from Fischer 344 rats. The tissues were cut into small pieces and cultured for up to 90 days after direct transduction with adenoviral BMP-2 or green fluorescence protein vectors. Different vector doses were applied, and proliferation, long-term BMP-2 production, and osteogenic differentiation of the 3 different tissues were investigated in vitro. Muscle with fascia produced the largest amounts of BMP-2. Expression of the transgene was detected for up to 90 days. Proliferation was reduced with increased vector doses. Muscle with fascia showed a higher potential for osteogenic differentiation than fat, but it was not improved as compared to muscle alone. A dose of 4×10^8 plaque forming units of the adenoviral BMP-2 vector appeared to be the optimal dose for transduction of muscle with fascia. Because muscle with fascia produced higher amounts of BMP-2 as compared to muscle alone or fat tissue grafts, showing a high potential for osteogenic differentiation, it might represent an improved osteo-regenerative implant facilitating endogenous repair. Future studies should investigate the effect of muscle with fascia transduced with 4×10^8 plaque forming units on bone healing in vivo.

KEYWORDS

bone regeneration, fascia, fat tissue, gene therapy, muscle tissue, osteogenic differentiation

1 | INTRODUCTION

Delayed fracture healing, non-unions, and large osseous defects remain great clinical challenges for surgeons around the world. In an ageing population, the demand for effective treatment options for such problems will dramatically increase in the near future (Schacter & Leslie, 2017).

The biological stimulation of bone growth by bone morphogenetic proteins (BMPs) holds great promise and has been performed clinically (Alt et al., 2015). However, delivery of such osteogenic molecules needs to be improved because of their short biological half-life (Jung et al., 2017). In order to achieve sustained delivery of BMPs and thereby a long lasting bone regeneration stimulus, gene transfer

Abbreviations: Ad.BMP-2, adenoviral vector carrying the bone morhogenetic protein gene; Ad.GFP, adenoviral vector carrying the green fluorescent protein gene; ALP, alkaline phosphatase; BAC, bacterial artificial chromosome; BMP, bone morphogenetic protein; CMV, cytomegalovirus; DMEM, Dulbecco's modified eagles medium; ELISA, enzyme-linked immunosorbent assay; GAPDH, gadolinium phosphate dehydrogenase; GFP, green fluorescence protein; HEK, human embryonic kidney; OCN, osteocalcin; PBS, phosphate buffered saline; pfu, plaque forming units; WST-1, water-soluble tetrazolium-1

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technologies have been developed. A drawback of such cell-based gene-enhanced tissue engineering approaches is that they require the isolation and expansion of autologous progenitor cells in an external good manufacturing practice-facility making them costly and time-consuming (Aslan et al., 2006). Another issue is the need for two separate surgical procedures, which increases costs of the approach and risks for the patient. The complexity of such methods impedes the translation from bench to bedside (Betz, Betz, Harris, Vrahas, & Evans, 2008). Therefore, the goal must be the development of simple same day, ex vivo gene therapies, which can be performed during a single surgical procedure (Virk et al., 2011).

The use of tissue grafts instead of isolated cells enables us to develop ex vivo gene transfer protocols that are better suited to clinical application. Betz et al. have utilized muscle and subcutaneous fat tissue transduced with an adenoviral BMP-2 vector to repair large bone defects in rats (Betz et al., 2009; Betz et al., 2010). The tissue grafts were directly contacted with the gene transfer vector leading to the genetically modification of cells on the surface of the tissue fragments. The so called "gene-activated tissue grafts" represent biological, defect-filling implants delivering growth factor and stem cells to the bone lesion. The stem cells within the muscle and adipose tissue grafts reside in their natural niches, which might support their proper function and capacity for differentiation.

Fat tissue got increasingly popular for bone tissue engineering purposes as it is easy to harvest in large quantities and contains progenitor cells with a proven osteogenic differentiation capacity (Tevlin et al., 2016). Adipose tissue fragments, genetically modified to overexpress BMP-2, have been successfully employed in a rat critical-size bone defect model (Betz et al., 2010). The implantation of BMP-2 gene-activated fat led to fast and robust structural and functional healing of femoral defects. Moreover, in recent work, it could be demonstrated that human fat tissue can transdifferentiate into bone-like tissue (Bondarava et al., 2017).

Muscle is another candidate tissue that can serve as an osteo-regenerative implant. Skeletal muscle possesses intrinsic scaffolding properties, and its harvest is associated with low donor site morbidity (Ahmad, Akhtar, Rashidi, & Khurram, 2013). Huard and colleagues have shown that muscle tissue hosts stem cells that can differentiate into the osteogenic lineage in vitro and in vivo (Gao et al., 2013; Wright et al., 2002). Recent studies exploring the potential of gene-activated muscle tissue grafts for bone repair could demonstrate that BMP-2 producing muscle implants rapidly heal large bone defects in rats (Betz et al., 2009). Interestingly, implantation of genetically modified muscle led to similar biomechanical stability and bone volume as compared to bone grafting (Betz et al., 2013).

The fascia of skeletal muscle consists of fibrous connective tissue containing fibroblasts. Only very little is known about the potential of fascia cells to regenerate other tissues. Recent work characterized fascia-derived cells in terms of their surface marker profile, proliferation rate, and chondrogenic potential (Li et al., 2011). The findings of this study suggest that the nonmyogenic cells of fascia have a strong chondrogenic potential and may contribute to ectopic bone formation in muscle via endochondral ossification. Our hypothesis was that fascia contains a larger number of cells on the surface as compared to muscle and fat resulting in improved transduction efficiencies and larger amounts of osteogenic molecules delivered to a site of bone injury. Moreover, an advantage for clinical use may be that fascia has favourable physical properties allowing a more stable fixation within a lesion, for example, by suturing.

Hence, the aim of the present in vitro experiment was to evaluate the potential of muscle with fascia to serve as a BMP-2 delivering osteo-regenerative implant in comparison to muscle alone and fat tissue. In addition, the optimal dose of an adenoviral BMP-2 vector for transduction of muscle with fascia was investigated.

2 | MATERIALS AND METHODS

2.1 | Vector production

Two different, first generation, serotype 5, E1, E3 deleted adenoviral vectors were used in this study: Ad.GFP (green fluorescent protein) and Ad.BMP-2 (bone morphogenetic protein-2). The vectors were constructed as reported previously (Betz et al., 2013) (Sirion Biotech GmbH, Martinsried, Germany). In summary, the *transgene* (GFP or BMP-2) coding regions were amplified by PCR and subcloned into the shuttle vector pO6-A5-CMV. The CMV-*transgene* portion of pO6A5-CMV-*transgene* was transferred in a BAC vector containing the genome of an Ad5-based E1/E3 deleted vector. Finally, HEK-293 cells were transfected with the adenoviral DNA, and amplified viral vector particles were chromatographically purified with ViraBind™ Adenovirus Purification Kit (Cell Biolabs Inc., San Diego, USA).

2.2 | Harvest of tissues, transduction, and culture system

Subcutaneous fat, muscle tissue, and muscle with fascia were harvested from the hind limbs of two donor animals. Small fragments of each tissue type were created using 4-mm dermal biopsy punches (pfm medical, Cologne, Germany). The round tissue pieces were then placed in 24-well plates and transduced with the adenoviral vectors carrying the BMP-2 or GFP cDNA using various concentrations ranging from 1×10^7 to 1×10^8 pfu for fat and muscle, and 1×10^7 to 1×10^9 pfu for muscle with fascia. Aliquots of 10 µl of the adenoviral vectors diluted in Dulbeccos's modified Eagle's medium (DMEM; BiochromeAG, Berlin, Germany) were added to the surface of the tissue fragments. Subsequently, the tissues were stored in an incubator (37 °C, 5% CO_2) for 1 hr. Four tissue fragments per well were cultured in a 24-well plate with 1 ml of DMEM (BiochromeAG, Berlin, Germany) for up to 90 days. Culture medium was changed every third day.

2.3 | Imaging of GFP-transduced tissue fragments under laser-scanning confocal microscopy

Forty-eight hours after transduction, adenoviral GFP-transduced tissue fragments were rinsed with phosphate buffered saline and then placed on a histological glass. The samples were imaged using an inverted laser-scanning confocal microscopy system (LSM 880;

Zeiss, Jena, Germany) with a 5× air objective (Zeiss, 0.25 NA, WD = 12.5 mm).

2.4 | Quantitative analysis of adenoviral GFP-transduction efficiency

Quantitative analysis of adenoviral GFP-transduction efficiency was performed using the Image J software v.1.6 (NIH). The area occupied by GFP-positive cells and the total area of the tissue disc were established. Values were expressed as a mean percentage of GFP-positive cells occupied area within the total area of tissue discs.

2.5 | Proliferation determined by water-soluble tetrazolium-1 (WST-1) assay

The ability of the cells of the tissues to proliferate was evaluated applying a WST-1 reagent (Roche, Mannheim, Germany) after 1, 2, 3, and 4 weeks. To perform the assay, the culture medium was discarded, and fresh medium was mixed with WST-1 at a ratio of 10:1(v/v). The tissue samples were then incubated together with the medium-WST-1 mixture for 2 hr at 37 °C in 5% CO_2. Subsequently, 100 µl of the mixture was transferred to a 96-well culture plate reading the absorbance at 450 nm with a Synergy HT microplate reader using the Gen 5 2.03 software (BioTek, Bad Friedrichshall, Germany).

2.6 | BMP-2 production measured by an enzyme-linked immunosorbent assay (ELISA)

The amounts of BMP-2 that were produced by the three different tissue types were determined using ELISA (Quantikine, BMP-2 Immunoassay, R&D Systems, Wiesbaden, Germany). Supernatants of tissue cultures were harvested every third day and collected over 30 days for fat and muscle and for up to 90 days for muscle with fascia. BMP-2 content in supernatants was measured according to the manufacturer's instructions. Absorbance was read using a Synergy HT microplate reader and the Gen 5 2.03 software (BioTek, Bad Friedrichshall, Germany).

2.7 | Quantitative real-time PCR analysis of osteogenic differentiation

Gene expression of alkaline phosphatase (*ALP*) and osteocalcin (*OCN*) was analysed by quantitative real-time PCR after 2 and 4 weeks. Tissues were frozen in liquid nitrogen and homogenized with a Mikro-Dismembrator S (Sartorius, Goettingen, Germany). The homogenate was then resuspended in 1 ml of QIAzol Lysis Reagent (Qiagen, Gemany), mixed with chloroform and centrifuged. Then the upper aqueous phase was collected and mixed with isopropanol. After centrifugation, total RNA pellet was rinsed with 75% ethanol, air-dried and re-dissolved in RNase-free water. The concentration and purity of total RNA were determined with a spectrophotometer (NanoDropTM Lite, Thermo Scientific, Waltham, USA). A thermocycler (T100, Bio-Rad, Germany) was used for cDNA synthesis (15 min at 42 °C). The reaction mixture contained 300 ng of total RNA, Quantiscript reverse transcriptase and RT primer mix from QuantiTect Reverse Transcription-Kit (Qiagen, Hilden, Germany) and buffers, diluted with RNase-free water to a total volume of 20 µl. The FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) and the Light Cycler 96 (Roche Diagnostics, Mannheim, Germany) were used for amplification of cDNA. The following reaction mixture at a final volume of 10 µl was used: 2.5 µl of cDNA, 0.5 µM of primer, 5 µl FastStart Essential DNA Green Master, and 2 µl RNase free water. The primers were purchased from Qiagen (Hilden, Germany). PCR reaction conditions were 10-min preincubation at 95 °C, 10-s denaturation at 95 °C, followed by 10-s annealing at 60 °C and 15-s extension at 72 °C for 40 cycles. Amplification was performed in triplicates. The $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative gene expression between samples using *GAPDH* as a housekeeping gene.

2.8 | Histological evaluation of calcium deposition within tissue fragments

After 4 weeks of tissue culture, tissue fragments were fixed in 4% paraformaldehyde for 2 hr, dehydrated in graded ethanol, and embedded in paraffin. Paraffin sections of 10 µm were prepared, mounted on superfrost glass slides (Menzel, Braunschweig, Germany), stained with freshly prepared 40-mM alizarin red S (Sigma–Aldrich, St. Louis, USA) solution, pH 4.1 for 10 min at RT, and finally washed with phosphate buffered saline. Histological samples were dehydrated with 100% ethanol and then cleared in Rothistol (Roth, Germany). After mounting the samples with DPX (Merck, Germany), the slides were analysed and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Jena, Germany) under a microscope (Axioscope 40, Carl Zeiss, Jena, Germany) using the Axiovision 40 software (Carl Zeiss, Jena, Germany).

2.9 | Statistical analysis

Microsoft Excel 2010 and Prism 5.02 software (GraphPad Software, San Diego, USA) were used for analysis of data. The results were presented as mean ± standard error. Nine samples per group were used for all assays. Measurements were performed in triplicate. In order to detect statistical differences, we applied the Mann–Whitney U-test or Kruskal–Wallis test, depending on the number of compared groups. Statistical significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$.

3 | RESULTS

3.1 | Imaging of GFP-transduced tissue fragments under laser-scanning confocal microscopy

Muscle with fascia revealed the highest number of green fluorescent cells after transduction with different doses of an adenoviral vector carrying the GFP cDNA. Muscle alone showed a well-known, strong background signal but less transduced cells after contact with 1×10^7, 5×10^7, or 1×10^8 pfu. Fat tissue samples displayed the lowest number of transduced cells. It was possible to further increase GFP expression in muscle with fascia with increasing doses of viral vector. Transduction with 1×10^9 pfu resulted in the strongest signal under laser scanning confocal microscopy indicating a very high level of GFP gene expression (Figure 1).

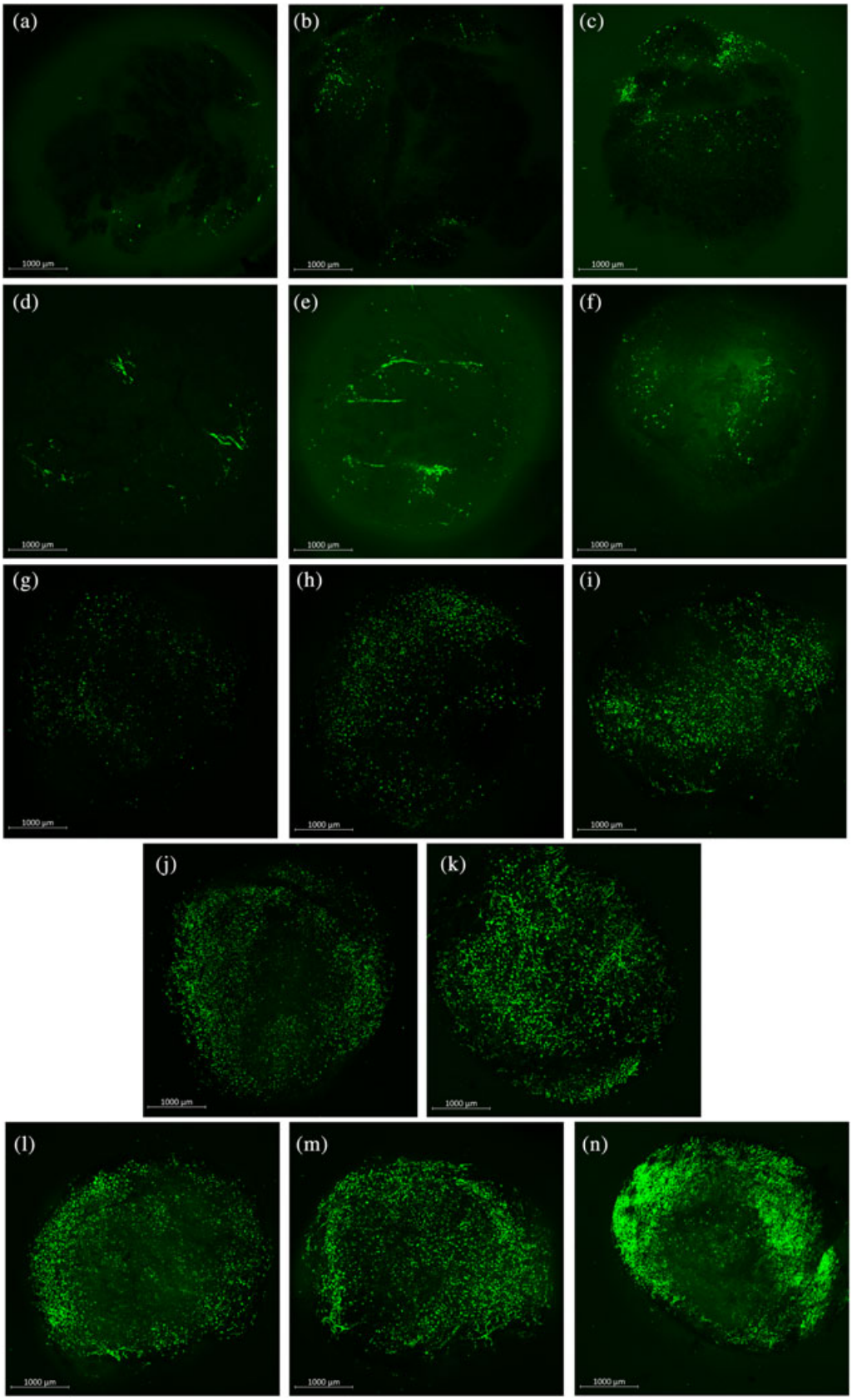

FIGURE 1 Imaging of green fluorescent protein (GFP) transduced tissue fragments under laser-scanning confocal microscopy. Adenoviral GFP-transduced tissue fragments were imaged with an inverted laser-scanning confocal microscopy system 48 hr after transduction. Displayed is a comparison of fat tissue, muscle tissue, and muscle with fascia transduced with 1×10^7 (a: fat; d: muscle; g: muscle with fascia), 5×10^7 (b: fat; e: muscle; h: muscle with fascia), or 1×10^8 (c: fat; f: muscle; i: muscle with fascia) plaque forming units (pfu) of an adenoviral GFP vector. Additionally, muscle with fascia was transduced with 2×10^8 (j), 4×10^8 (k), 6×10^8 (l), 8×10^8 (m), and 1×10^9 (n) pfu of adenoviral GFP vector. Scale bar = 1,000 µm [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Quantitative analysis of adenoviral GFP-transduction efficiency

Quantitative analysis further substantiated that, after transduction with the same dose of Ad.GFP, muscle with fascia showed a significantly larger area of green fluorescent cells compared to muscle or fat. Muscle showed a significantly larger area of green fluorescent cells than fat. All three tissues showed an increasing area of GFP-positive cells with increased doses of viral vector ranging from 1×10^7 to 1×10^8 pfu (Figure 2a). A further increase of green fluorescent cell numbers was found when higher doses of GFP vectors were applied to muscle with fascia ranging from 2×10^8 to 1×10^9 pfu. Transduction with 1×10^9 pfu resulted in the largest area of GFP-positive cells, which was significantly higher than with all other doses. 2×10^8 pfu induced the smallest area of green flourescent cells, which is significantly smaller than the ones achieved with all other doses. No significant differences were detected between doses ranging from 4×10^8 to 8×10^8 pfu (Figure 2b).

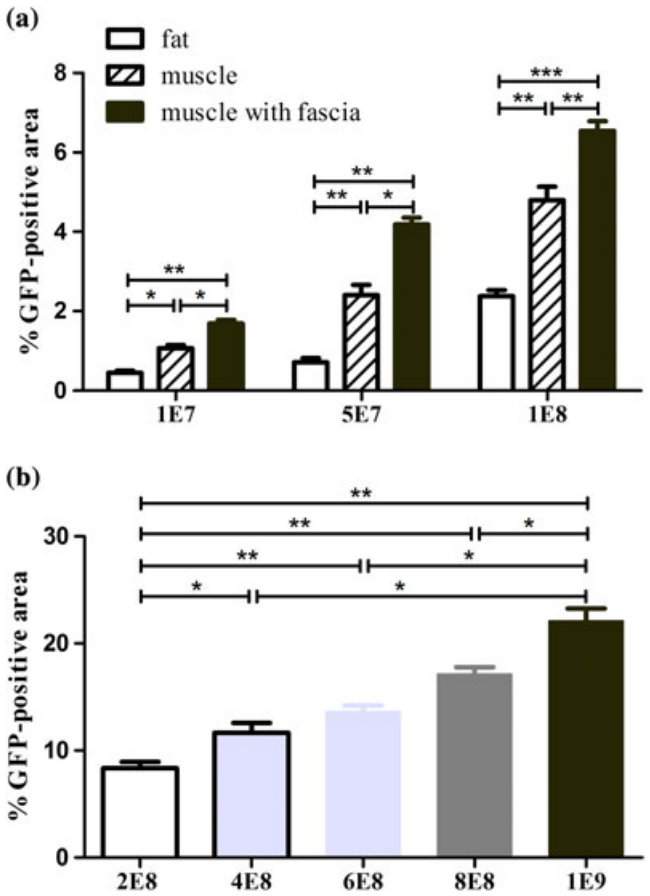

FIGURE 2 Quantitative analysis of adenoviral GFP-transduction efficiency. Ad.GFP-transduction efficiency was analysed using Image J software v.1.6, and the comparison was calculated utilizing Prism software 5.02. (a) % GFP-positive area of the three different tissues with doses ranging from 1×10^7 to 1×10^8 pfu. (b) % GFP-positive area after transduction of muscle with fascia with doses ranging from 2×10^8 to 1×10^9 pfu. Values are presented as mean ± standard error. The level of significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Proliferation determined by WST-1 assay

The WST-1 assay revealed that proliferation rate was reduced after transduction with the adenoviral BMP-2 vector as compared to unmodified tissue. Increasing doses of viral vector led to a reduced proliferation rate. Proliferation of fat tissue was significantly lower as compared to muscle alone and muscle with fascia. Proliferation rate of muscle with fascia was higher than muscle alone over the 4-week period, but a significant difference was detected only for the second week of culture ($p < .01$), indicating an increased cell growth in muscle with fascia. At 4 weeks, proliferation rate peaked for muscle alone and muscle with fascia. Proliferation rates as determined for muscle with fascia after 4 weeks in culture was as follows: unmodified tissue = 129.70 ± 5.51; 1×10^7 = 115.80 ± 6.24; 5×10^7 = 72.47 ± 2.55; $1x10^8$ = 63.74 ± 2.94. Lower proliferation rates were observed for muscle alone at 4 weeks: unmodified tissue = 124.60 ± 5.83; 1×10^7 = 103.40 ± 3.96; 5×10^7 = 66.80 ± 6.27; 1×10^8 = 58.43 ± 4.79. The lowest proliferation rate was noted for fat tissue at 4 weeks: unmodified tissue = −0.02 ± 0.02; 1×10^7 = −0.05 ± 0.02; 5×10^7 = −0.06 ± 0.02; 1×10^8 = −0.26 ± 0.09 (Figure 3).

3.4 | BMP-2 production measured by an ELISA

Measurement of BMP-2 concentrations by ELISA demonstrated that all three types of tissue produced BMP-2 for at least 30 days in vitro when transduced with doses of 1×10^7, 5×10^7, or 1×10^8 pfu. The increase of the dose led to increased amounts of produced BMP-2. Muscle with fascia produced the highest levels of BMP-2, and fat produced the lowest levels. The peak of BMP-2 content was seen at Day 9 for all three types of tissue. At Day 9, transduction with 1×10^8 pfu led to 38.49 ± 1.89 ng/ml of BMP-2 produced by muscle with fascia, which was significantly higher than 24.37 ± 1.82 ng/ml of BMP-2 produced by muscle alone ($p < .01$) and 7.66 ± 0.58 ng/ml of BMP-2 produced by fat ($p < .001$). When transduced with higher doses of adenoviral BMP-2 vector ranging from 2×10^8 to 1×10^9 pfu, muscle with fascia produced BMP-2 at consistent high levels until Day 30. At Day 30, the BMP-2 concentrations in supernatants of muscle with fascia after transduction with various doses of vector were as follows: 1×10^7:6.56 ± 0.62 ng/ml; 5×10^7:10.44 ± 0.82 ng/ml; 1×10^8:14.11 ± 1.70 ng/ml; 2×10^8:13.81 ± 0.82 ng/ml; 4×10^8:29.28 ± 1.41 ng/ml; 6×10^8:22.81 ± 1.27 ng/ml; 8×10^8:26.60 ± 0.44 ng/ml; 1×10^9:27.53 ± 1.60 ng/ml. Concentrations in supernatants of muscle alone on Day 30 were 5.38 ± 0.63 ng/ml (1×10^7), 7.84 ± 0.35 ng/ml (5×10^7), and 10.74 ± 0.25 ng/ml (1×10^8); concentrations of BMP-2 in supernatants of fat tissue were 1.23 ± 0.06 ng/ml (1×10^7), 1.29 ± 0.06 ng/ml (5×10^7), and 1.51 ± 0.05 ng/ml (1×10^8). The highest concentration during the whole culture period was seen after transduction of muscle with fascia with 4×10^8 pfu at Day 18 (43.23 ± 2.31 ng/ml). Comparatively, transduction with 4×10^8 and 1×10^9 pfu induced a significantly higher BMP-2 expression than with other doses during the first 30 days, whereas a significant difference between 4×10^8 and 1×10^9 pfu was observed only at Days 6 and 18 ($p < .05$). The assay also revealed that muscle with fascia secretes BMP-2 for at least 90 days in vitro (Figures 4 and 5).

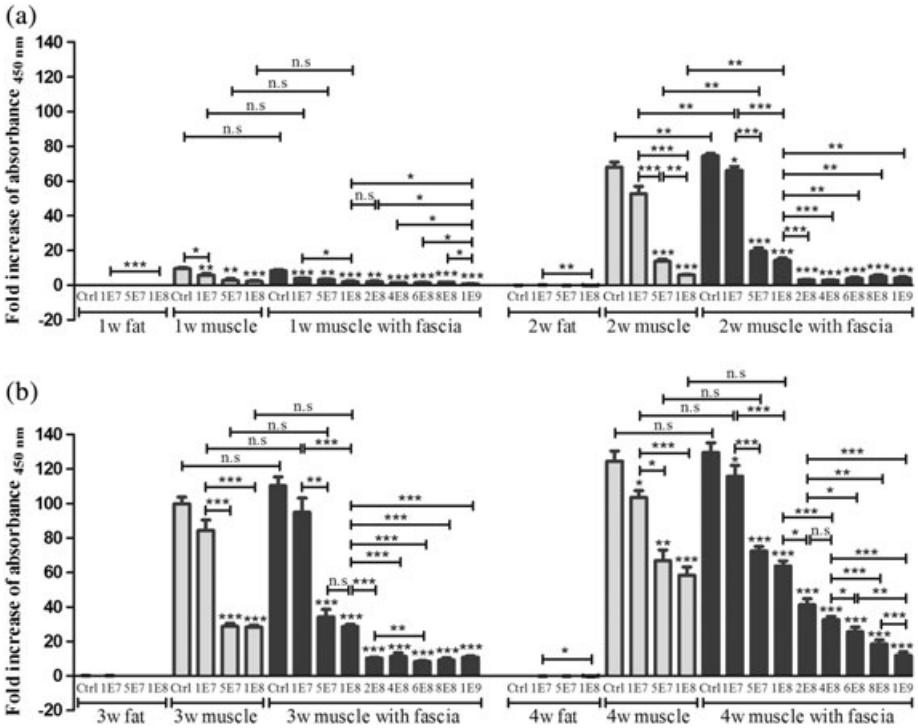

FIGURE 3 Proliferation determined by water-soluble tetrazolium-1 (WST-1) assay. After 1, 2, 3, and 4 weeks of tissue culture, the ability of the three types of tissue to proliferate was evaluated using a water-soluble tetrazolium-1 (WST-1) assay. (a) Proliferation after 1 and 2 weeks. (b) Proliferation after 3 and 4 weeks. Nine samples per group were analysed. Measurements were carried out in triplicate. Values are presented as mean ± standard error. The level of significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$

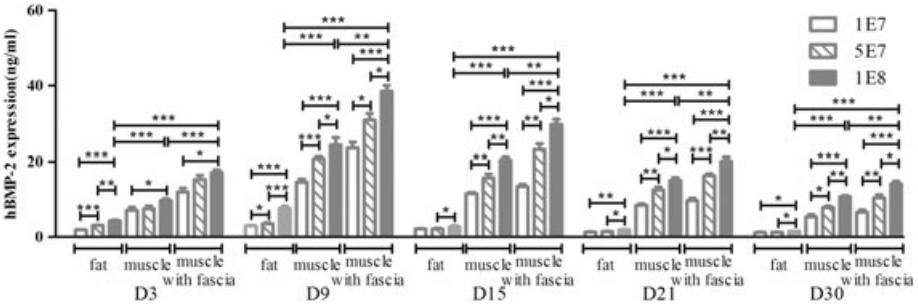

FIGURE 4 BMP-2 produced by fat, muscle, and muscle with fascia determined by enzyme-linked immunosorbent assay (ELISA). After transduction of fat, muscle, and muscle with fascia with 1×10^7, 5×10^7, or 1×10^8 plaque forming units of an adenoviral BMP-2 vector, BMP-2 concentrations in supernatants were determined by ELISA over 30 days. Nine samples per group were analysed. Measurements were carried out in triplicate. Values are presented as mean ± standard error. The level of significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$

3.5 | Quantitative real-time PCR analysis of osteogenic differentiation

The analysis of gene expression by real-time PCR demonstrated that transduction with an adenoviral BMP-2 vector led to overexpression of the early osteogenic differentiation marker alkaline phosphatase (*ALP*) and the late bone marker osteocalcin (*OCN*) in all three types of tissue.

The highest *ALP* mRNA expression level was noted for muscle tissue alone after transduction with 1×10^7, 5×10^7, and 1×10^8 pfu at two (11.16 ± 1.03; 19.46 ± 2.68; 43.98 ± 16.34) and four (35.29 ± 1.70; 93.69 ± 2.48; 201.00 ± 12.94) weeks. *ALP* expression levels in muscle with fascia were significantly lower with 5.43 ± 0.64 ($p < .05$), 7.21 ± 0.78 ($p < .01$), and 19.40 ± 3.44 ($p < .05$) at 2 weeks, and 24.26 ± 2.13 ($p < .05$), 51.14 ± 2.29 ($p < .001$), and 97.98 ± 10.11 ($p < .01$) at 4 weeks. Fat tissue showed the lowest *ALP* levels. The dose of 1×10^8 pfu induced a higher *ALP* expression in all three types of tissue than the two lower doses, at 2 and 4 weeks. Fat tissue showed a higher *ALP* expression at 2 weeks than at 4 weeks, whereas *ALP* expression in muscle with fascia and muscle alone peaked after 4 weeks of culture. Transduction of muscle with fascia with additional doses of 2×10^8 to 1×10^9 pfu resulted in a further increase of *ALP* expression (26.19 ± 2.10; 48.76 ± 6.51; 32.12 ± 1.21; 36.18 ± 5.28; 38.17 ± 6.30) respectively at 2 weeks, and 303.20 ± 16.67; 472.40 ± 19.61; 209.30 ± 22.16; 217.30 ± 9.15; 250.80 ± 10.80 respectively at 4 weeks). The highest level of *ALP*

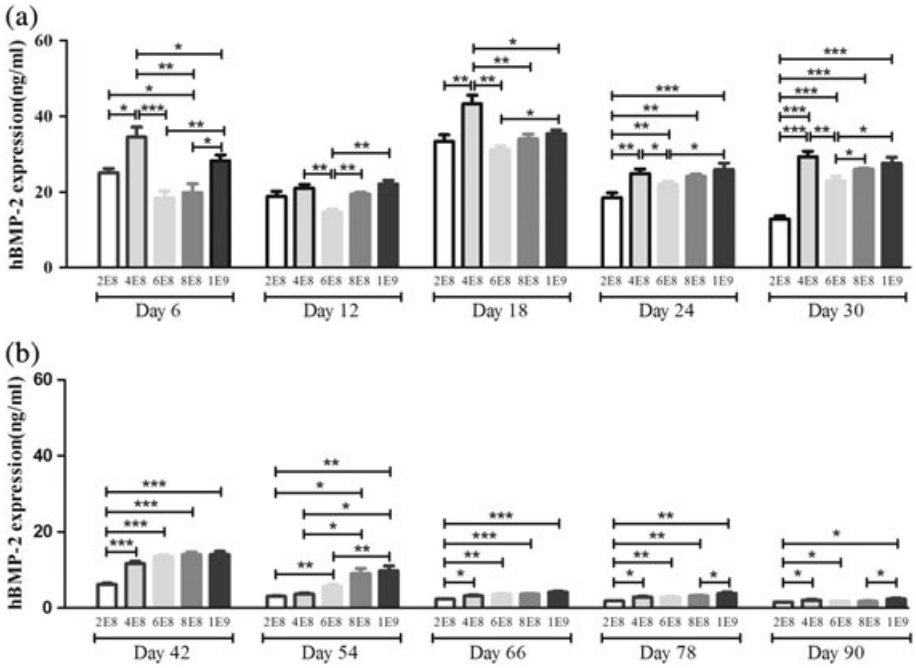

FIGURE 5 BMP-2 produced by muscle with fascia after transduction with higher doses of adenoviral vector determined by ELISA. Muscle with fascia was transduced with 1×10^8, 2×10^8, 4×10^8, 6×10^8, 8×10^8, and 1×10^9 plaque forming units of an adenoviral BMP-2 vector. Concentrations of BMP-2 in supernatants were determined by ELISA over 90 days. (a) Measurements from Day 6 until Day 30. (b) Measurements from day 42 until day 90. Nine samples per group were analysed. Measurements were carried out in triplicate. Values are presented as mean ± standard error. The level of significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$

mRNA expression in muscle with fascia was noted for the dose of 4×10^8 pfu of the adenoviral BMP-2 vector especially at 4 weeks ($p < .001$).

After transduction with 1×10^7, 5×10^7, and 1×10^8 pfu, the highest *OCN* mRNA expression levels at 2 weeks were noted for muscle alone (5.51 ± 0.23, 11.71 ± 0.45, and 14.92 ± 0.49, respectively), which was significantly higher than for muscle with fascia at 5×10^7 (6.58 ± 0.50, $p < .001$) and 1×10^8 (7.32 ± 0.39, $p < .001$). After 4 weeks, however, the *OCN* expression levels after transduction with 1×10^7, 5×10^7, and 1×10^8 pfu were not significantly different between muscle with fascia and muscle alone. Fat tissue showed the lowest *OCN* expression at 2 weeks (0.15 ± 0.02; 0.25 ± 0.02; 0.55 ± 0.06, respectively) and 4 weeks (0.25 ± 0.02; 0.29 ± 0.02; 0.51 ± 0.03, respectively), and a significant difference was noticed in comparison to muscle alone ($p < .001$) and muscle with fascia ($p < .001$). In all three types of tissue, the dose of 1×10^8 pfu induced a higher *OCN* expression than 1×10^7 and 5×10^7 pfu, at 2 and 4 weeks. The higher doses of 4×10^8 and 1×10^9 pfu used for transduction of muscle with fascia resulted in the highest levels of *OCN* mRNA expression in muscle with fascia at 2 weeks (4×10^8:27.24 ± 1.29; 1×10^9:26.20 ± 1.76) and 4 weeks (4×10^8:15.61 ± 1.03; 1×10^9:17.16 ± 2.13), and no significant difference was observed between 4×10^8 and 1×10^9 (Figure 6).

3.6 | Histological evaluation of calcium deposition within tissue fragments

Calcium deposition was detected by alizarin-red staining of tissue sections after 4 weeks of tissue culture. BMP-2 transduction induced mineralization of muscle with fascia and muscle alone, but not fat tissue. Calcium deposition was visible in the superficial zones of tissue discs. Unmodified control tissue samples did not exhibit any mineralization. When transduced with 1×10^8 pfu, muscle with fascia and muscle alone showed a similar extension of mineralization. Most calcium deposition in muscle with fascia was detected after transduction with a dose of 4×10^8 pfu of the adenoviral BMP-2 vector (Figure 7).

4 | DISCUSSION

It is well documented that genetically modified stem cells can be used to deliver growth factors to the site of tissue injuries. In particular, there is a large number of studies suggesting that stem cells derived from bone marrow, muscle, and adipose tissue are capable of inducing bone growth when transduced with an osteogenic gene (Lee et al., 2002; Lieberman et al., 1999; Peterson et al., 2005). In an attempt to simplify ex vivo gene therapy for tissue engineering, a novel strategy involving the use of gene-activated tissue grafts was developed (Betz et al., 2009; Betz et al., 2010). This method allows to effectively restore bone defects in preclinical animal models utilizing autologous muscle or fat tissue as biological, three-dimensional, degradable scaffolds delivering progenitor cells and osteogenic molecules to an osseous lesion.

In order to further improve this technology, we identified fascia as another interesting tissue structure that could serve as an osteoregenerative drug delivery system supporting endogenous repair of bone. Fascia is a sheet of connective tissue consisting primarily of collagen with a large number of fibroblasts residing on the surface. Our data indicate that these cells can be transduced effectively when applying an adenoviral vector to the surface of fascia. In contrast, there

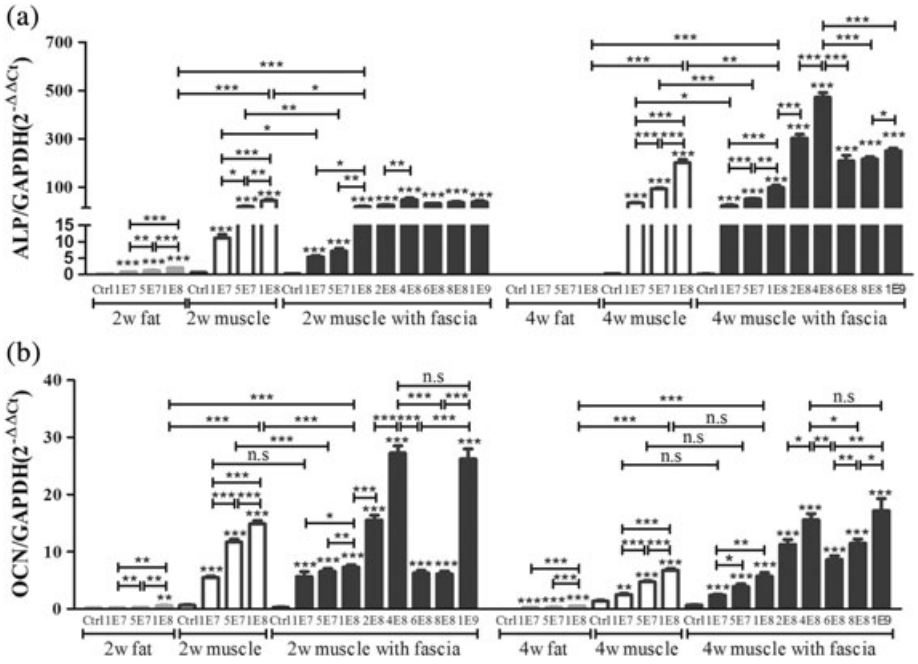

FIGURE 6 Quantitative real-time PCR analysis of osteogenic differentiation. Expression levels of osteogenic markers after transduction of fat, muscle, and muscle with fascia with various doses of an adenoviral BMP-2 vector was analysed by quantitative real-time PCR at 2 and 4 weeks. (a) Alkaline phosphatase (*ALP*) mRNA expression. (b) Osteocalcin (*OCN*) mRNA expression. Expression levels were normalized to mRNA levels for GAPDH. Nine samples per group were analysed. Measurements were carried out in triplicate. Values are presented as mean ± standard error. The level of significance was indicated by * for $p < .05$, ** for $p < .01$, and *** for $p < .001$

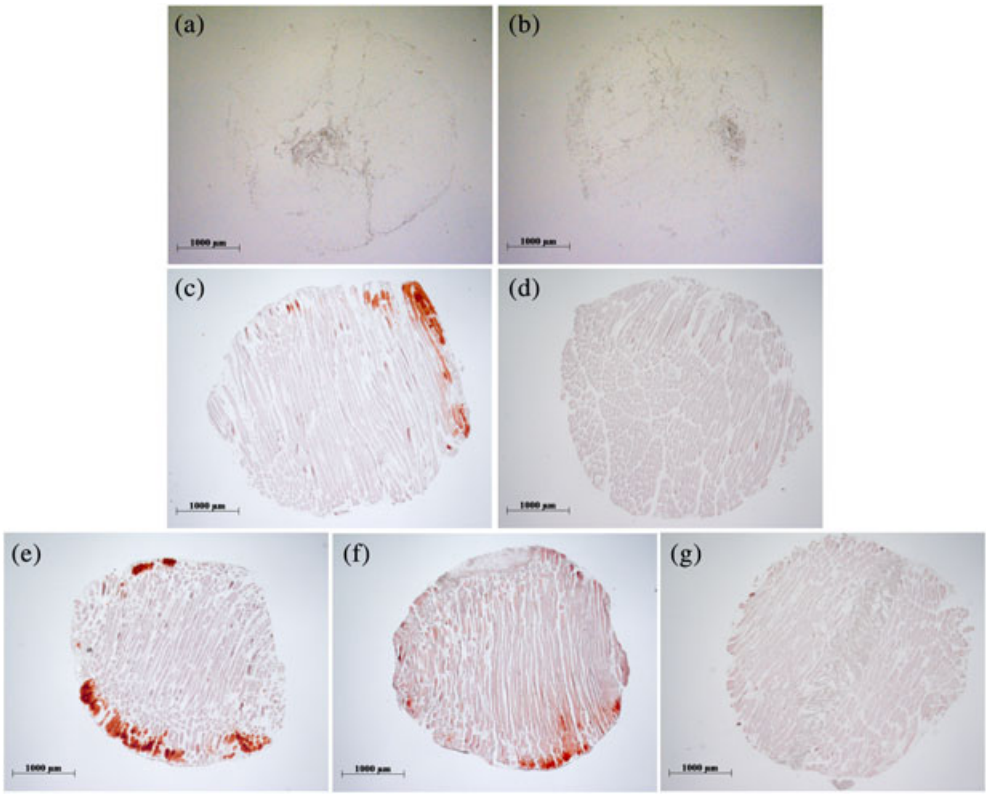

FIGURE 7 Histological evaluation of calcium deposition within tissue fragments. After 4 weeks of tissue culture, histological sections of fat, muscle, and muscle with fascia were stained with alizarin red in order to detect calcium deposition. Representative cross-sections of samples transduced with 1×10^8 plaque forming units (pfu) of an adenoviral BMP-2 vector and unmodified control samples are shown. Transduction with 4×10^8 pfu induced the strongest osteogenic response in muscle with fascia. Fat: (a) 1×10^8 pfu, (b) unmodified control; muscle: (c) 1×10^8 pfu, (d) unmodified control; muscle with fascia: (e) 4×10^8 pfu, (f) 1×10^8 pfu, (g) unmodified control. Scale bar = 1,000 μm [Colour figure can be viewed at wileyonlinelibrary.com]

are fewer cells on the surface of muscle and fat tissue fragments that can be transduced with the same adenoviral vector leading to a reduced production of GFP and BMP-2. This suggests that fascia can enhance delivery of the transgene leading to larger amounts of osteogenic growth factor locally as shown by the presented data generated by ELISA.

Wong et al. explored the characteristics of rat fascia-derived stem cells (Wong et al., 2015). Cells were isolated from rat fascia and stimulated to differentiate into the chondrogenic, osteogenic, and adipogenic lineage. The results from this study show that rat fascia-derived stem cells possess high chondrogenic but a lower osteogenic and adipogenic potential as compared to bone marrow-derived stem cells and adipose-derived stem cells. In our present study, the potential of muscle with fascia to differentiate into the osteogenic lineage was significantly higher than that of adipose tissue. Importantly, it must be noted that no osteogenic supplements were added to the culture medium as we intended to solely study the effect of BMP-2 expression levels and its effects on the different tissues. This, and the fact that we investigated the behaviour of tissue fragments instead of isolated cells, might explain why the results of other differentiation studies vary.

When preparing the tissue fragments, we took great care that no fascia was attached to the "muscle alone samples." We cannot completely exclude that there was any deep fascia within the inner parts of the "muscle alone" fragments. However, the important difference between "muscle alone" and "muscle with fascia" for transduction was the surface of the fragments. "Muscle with fascia" samples were completely covered on one side with fascia resulting in higher transduction efficiency. The question whether the osteogenic differentiation capacity or the potential to deliver high levels of transgene is more important for effective bone regeneration can only be answered by future in vivo experiments. Interestingly, recent in vitro studies could prove that human fat tissue (Bondarava et al., 2017) and human muscle tissue (Miao et al., 2017) can transdifferentiate into mineralized tissue with bone-like characteristics. These results suggest that the grafted gene-activated tissue fragments used in the experiments of Betz and colleagues (Betz et al., 2009; Betz et al., 2010) contributed to the formation of new bone in vivo through transdifferentiation and not only through the local effects of the delivered BMP on the environment of the lesion. The secreted BMPs seem to induce bone growth via combined effects of graft transdifferentiation as well as recruitment, proliferation, and differentiation of progenitor cells residing in the proximity of the osseous defect. For these reasons, it becomes obvious that much effort must be put into the development of appropriate drug delivery systems. With respect to BMPs and bone repair, such improved delivery methods are of particular interest because large amounts of recombinant human BMP-2 applied clinically led to adverse side effects in humans (James et al., 2016). Instead of introducing milligrams of BMP-2 into the body, gene transfer leads to a local production of only nanogram quantities of BMP-2 within the bony lesion.

The harvest and transplantation of fascia alone or in combination with muscle is a common procedure frequently performed by plastic and reconstructive surgeons in order to cover soft tissue defects and reconstruct deformities (Lee et al., 2016; Nuri, Ueda, & Yamada, 2016; Veyrat, Verillaud, Herman, & Bresson, 2016). Based on the results of our present work, we hypothesize that muscle with fascia genetically modified to overexpress BMP-2 could also serve as an osteo-regenerative implant repairing large bone defects. The combination of muscle with fascia might be an advantageous graft for bone repair because it delivers larger amounts of BMP-2 as compared to fat and muscle alone while still showing a high osteogenic differentiation capacity. The dose of 4×10^8 pfu of an adenoviral BMP-2 vector seems to be optimal for transduction leading to both high levels of BMP-2 production and effective osteogenic differentiation.

The promising data of the present in vitro study encourage to carry out an in vivo experiment evaluating the effect of BMP-2 gene-activated muscle with fascia on the regeneration of large bone defects in a well-established preclinical model.

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CONFLICT OF INTEREST

No conflict of interest exists for all other authors.

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Summary

Current cell-based strategies for the treatment of large segmental bone defects remain far from ideal. The extraction and expansion of autologous stem cells, as well as the complexity of scaffold fabrication are significant obstacles for their clinical application. Previous studies have demonstrated that bone morphogenetic protein-2 (BMP-2) genetically engineered fat or muscle tissue could achieve sustained delivery of BMP-2 and bone healing *in vivo*. Aim 1 of my thesis was to investigate whether the fat tissue graft is capable of transdifferentiation into bone or merely serves as a protein delivery vehicle in previous transduction studies (Publication 1). Part 2 of my thesis explored BMP-2 expression profiles in gene activated fat tissue, muscle tissue, and muscle with fascia tissue under various transduction dosages, aiming to optimize this tissue-based molecular signals delivery system (Publication 2).

In the first study, human fat tissue fragments were cultured in normal growth medium or osteogenic medium with or without the stimulus of recombinant BMP-2, BMP-7 or BMP-9 for 4 weeks. Calcium deposition analysis, RT-PCR, and immunofluorescence of bone markers were performed to detect osteogenic differentiation in fat tissue. Osteogenic differentiation was induced when fat tissue fragments were cultured in osteogenic medium, and the addition of recombinant BMPs further increased calcium deposition, *ALP* and *OCN* mRNA expressions, as well as bone marker protein expressions. Our study demonstrated that fat tissue is capable to undergo osteogenic transdifferentiation without stem cells isolation. This finding encourages the conversion of cell-based tissue engineering approaches to tissue-based approaches, which will greatly simplify current bone regeneration strategies.

In the second part of my thesis (Publication 2), fat tissue, muscle tissue, and muscle with fascia tissue were harvested from rats and transduced with different adenoviral vector doses encoding human BMP-2 (Ad.hBMP-2). The proliferation, BMP-2 expression and osteogenic differentiation were analyzed by WST-1, ELISA and RT-PCR. The comparison of fat tissue,

muscle tissue and muscle with fascia tissue transduced by Ad.hBMP-2 under the same dose revealed, that muscle with fascia exhibited the highest BMP-2 expression. The transduction dose at 4×10^8 plaque forming units (pfu) appeared to be optimal for transduction of muscle with fascia.

Fascia of muscle remarkably enhanced BMP-2 protein expression after gene transduction, showing a high potential as an improved osteo-regenerative implant facilitating endogenous repair. Considering that 4×10^8 pfu has been indicated as the optimal dose for gene transduction, future *in vivo* studies should focus on investigating the osteogenic transdifferentiation capacity of muscle with fascia under such a transduction dose as well as the potential of human fat tissue fragments for the regeneration of bone defects.

Zusammenfassung

Aktuelle, zellbasierte Strategien zur Behandlung segmentaler Knochendefekte kritischer Größe bedürfen der weiteren Optimierung. Die Entnahme und Vermehrung autologer Zellen sowie die Komplexität der Herstellung von optimalen Trägermaterialien sind nach wie vor signifikante Hindernisse für den erfolgreichen Einsatz als klinische Anwendung. Vorhergehende Studien konnten zeigen, dass Fett- und Muskelgewebe, nach Transduktion mit Bone Morphogenic Protein-2 (BMP-2) Genen, zu einer ausreichend langen Expression des osteogenen Wachstumsfaktors führt, um Knochendefekte kritischer Größe *in vivo* zu regenerieren. Der erste Teil meiner Doktorarbeit beschäftigt sich mit der Frage, ob humanes Fettgewebe durch osteogene Stimulation in der Lage ist in Knochen oder knochenähnliches Gewebe zu transdifferenzieren oder ob das transduzierte Fettgewebe der früheren Studien vorwiegend als Wirkstofflieferant diente (Publikation 1). Teil 2 meiner Arbeit beschäftigt sich mit der Optimierung von gentransduziertem Fett-, Muskel- und Fasziengewebe . Hierbei war das Ziel, sowohl optimale Dosierung des Vektors als auch das am besten geeignete Gewebe zu identifizieren (Publikation 2).

Im ersten Teil meiner Arbeit (Publikation 1) wurden humane Fettgewebefragmente in normalen Zellkultur Medium sowie in osteogenem Medium allein oder mit zusätzlichem Stimulus durch die rekombinanten Wachstumsfaktoren BMP-2, BMP-7 oder BMP-9 über die Dauer von vier Wochen kultiviert. Die Bewertung der ostogenen Differenzierung erfolgte mittels Analyse der Kalziumeinlagerung sowie der Expression von Knochenmarkern mittels real-time PCR und Immunofluoreszenz. Osteogene Differenzierung konnte in den Fettgewebefragmenten, die in ostogenem Medium kultiviert wurden, nachgewiesen werden. Die Zugabe von rekombinanten BMP’s führte zu einem höheren Grad der osteogenen Differenzierung. Dies zeigte sich in höherer Kalziumeinlagerung sowie der verstärkten Expression der Knochenmarker. Das Ergebnis dieser Studie zeigt, dass humanes Fettgewebe

osteogen differenziert werden kann ohne eine vorherige Isolation und Kultivierung von Stammzellen durchzuführen und deutet darauf hin, dass ein Wechsel von einer zellbasierten Regeneration zu einer gewebebasierten Regeneration möglich ist. Dies würde eine Anwendung stark vereinfachen und die Chancen eines breiten klinischen Einsatzes deutlich verbessern.

Im zweiten Teil meiner Arbeit (Publikation 2) wurde Fett- und Muskelgewebefragmente mit und ohne Fasziengewebe von Ratten entnommen und anschließend mit verschieden adenoviralen Dosen mit dem BMP-2 Gen transduziert. Die Proliferation, BMP-2 Expression und osteogene Differenzierung wurden mit Hilfe eines WST-1 Tests, ELISA bzw. der real-time PCR untersucht. Der Vergleich der BMP-2 Expression von Fett-, Muskel- und Muskel mit Faziengewebefragmenten nach Transduktion mit der identischen Vektordosis ergab die höchste BMP-2 Expression in der Muskelgewebe mit Faszien Gruppe. Eine Vektordosis von 4×10^8 plaque forming units (pfu) stellte sich als optimale Dosierung heraus. Das Ergebnis zeigt das Potential von Muskelfazien für eine effektive Wirkstoffexpression zur Optimierung des Regenerationspotential von Gewebeimplantaten. Für weiterführende Studien sollte sowohl die als optimale Dosis ermittelte Vektormenge in Verbindung mit den Muskelfazienimplantaten sowie das Potential der humanen Fettgewebefragmente für die Knochendefektregeneration *in vivo* untersucht werden.

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