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REVIEW

Scaffold-free microtissues: differences from monolayer cultures and their potential in bone tissue engineering

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Abstract
Objectives Cell-based therapies for bone augmentation after tooth loss and for the treatment of periodontal defects improve healing defects. Usually, osteogenic cells or stem cells are cultivated in 2D primary cultures, before they are combined with scaffold materials, even though this means a loss of the endogenous 3D microenvironment for the cells. Moreover, the use of single-cell suspensions for the inoculation of scaffolds or for the direct application into an area of interest has the disadvantages of low initial cell numbers and susceptibility to unwanted cellular distribution, respectively.
Materials and methods We addressed the question whether an alternative to monolayer cultures, namely 3D microtissues, has the potential to improve osteogenic tissue engineering and its clinical outcome.
Results By contrast, to monolayer cultures, osteogenic differentiation of 3D microtissues is enhanced by mimicking in vivo conditions. It seems that the osteogenic differentiation in microtissues is enhanced by strong integrin–extracellular matrix interaction and by stronger autocrine BMP2 signaling. Moreover, microtissues are less prone to wash out by body fluids and allow the precise administration of large cell numbers.
Conclusion Microtissue cultures have closer characteristics with cells in vivo and their enhanced osteogenic differentiation makes scaffold-free microtissues a promising concept in osteogenic tissue engineering.
Clinical relevance Microtissues are particularly suitable for tissue engineering because they improve seeding efficiency of biomaterials by increasing the cell load of a scaffold. This results in accelerated osteogenic tissue formation and could contribute to earlier implant stability in mandibular bone augmentation.

Keywords Osteogenic tissue engineering · Cellular aggregates · Spheroids · 3D cell culture

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Background

The already high number of patients that need therapy for the treatment of critical-size bone defects will further increase in the future due to the rising age of the population. Scaffold materials that are used to bridge defects need to be combined with osteogenic cells to guarantee successful healing of the defect. Bone defects after trauma, infection, tumor resection, and osteoporosis, as well as defects of the cranio- and maxillofacial skeletal system caused by tooth loss and age-related atrophy of the jaw, are major clinical problems. Frequently, conventional methods for bone tissue regeneration, such as transplantation of autologous bone grafts, are not possible due to restricted availability of the bone or significant morbidity of the donor site [1]. Furthermore, there are significant disadvantages of alternative bone-filling materials, including infection and insufficient osseointegration. As an alternative, tissue engineering-based bone reconstruction therapies promise new therapeutic opportunities [2]. With the combination of scaffolds and growth

factors, cellular customized tissue-engineered bone grafts could be grown in vitro and implanted [3]. For a detailed review about bone regeneration by stem cell and tissue engineering in general please refer to the review of Z. Y. Zhang et al. [4] and for a review especially of the oral and maxillofacial region, please refer to the work of Z. Zhang [5]. Besides the significant progress in the field of bone tissue engineering and the already high potential of stem cell-based bone tissue engineering applications [4], findings about the importance of cell–cell and cell–matrix contacts in 3D cell cultures [6] have highlighted potentialities to further improve osteogenic tissue engineering. As a result, several 3D culture systems for multipotent stem cells and osteoblastic cells have been developed, which have led to the discovery of improved osteoblast differentiation in 3D compared to 2D cultures [7–12]. As a consequence, massive research has been performed to combine 3D-culture technologies with osteogenic tissue engineering. In the literature, these cell aggregates are referred to as cellular spheroids, micromasses, microspheres, or microtissues, the last being used in this review.

Methods for the production of microtissues and their advantages in different applications

Microtissue technology for osteogenic tissue engineering emerged from three different research fields, all dealing with cell agglomerates of different kinds of cells. For almost 20 years, the potential of multicellular spheroids as a 3D in vitro culture system has been used to study tumor biology. According to Kunz-Schughart et al. [13], "the growth of tumor cells as three-dimensional multicellular spheroids in vitro has led to important insights in tumor biology, since properties of the *in vivo* tumor such as proliferation or nutrient gradients can be studied under controlled conditions." Another research field dealing with microtissues or agglomerated cells is cartilage engineering. Chondrocytes are propagated in cell culture and are re-aggregated into microtissues [14]. This results in the formation of cartilage-like tissues by the addition of chondrogenic factors. Moreover, the chondrogenic differentiation of multipotent stem cells, like mesenchymal stem cells from human cord blood, is routinely performed by their agglomeration and the addition of chondrogenic factors [15]. The third research field dealing with cellular microtissues is developmental biology, where embryoid bodies (EB) are formed from embryonic stem cells (ESC). All three methods provided deep insight into the complexity but also into the potential of 3D cell cultures for bone tissue engineering. Spheroid culture in tumor biology provided knowledge about borders that are set to the technique by limited diffusion of nutrients and gases. Agglomerates in cartilage engineering demonstrated the potential of microtissues in the directed differentiation of tissue progenitors and multipotent stem cells. At last, microtissues in developmental biology provided evidence that pluripotent stem cells differentiate spontaneously into cells of different germ layer, just because of their culture in spheroids.

Several methods have been developed for the production of 3D tissues. In cartilage engineering multipotent stem cells, for example from adipose tissue or bone marrow, and cartilage-derived chondrocytes have been agglomerated by the addition of defined cell numbers to centrifuge tubes and centrifugation for a few minutes (Fig. 1a) [16, 17]. With this method, a pellet is generated that can be transferred into culture vessels, which provide a better medium supply than the centrifuge tube. Embryoid body formation for the differentiation of ESC can be achieved by using non-adherent plane culture surfaces or the hanging drop method (Fig. 1b) [18–20]. Both methods are suitable for generating EB by the attachment of single cells to each other. However, only the hanging drop method allows the generation of EBs from single-cell clones. A cell suspension is diluted until only one cell per drop of medium is left that is then placed on the surface of a culture plate that is then turned upside down. The surface tension of the medium holds the drop in position and the cell or the EB floats in the medium. On the

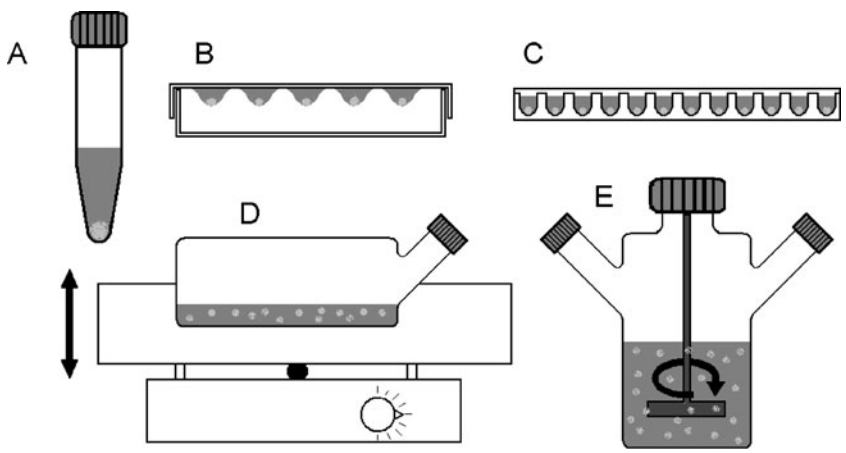

Fig. 1 Techniques for the production of microtissues. Generation of large microtissues by centrifugation in tubes (**a**), small microtissues and embryoid bodies for example from single-cell clones in hanging drops (**b**), microtissues in variable but defined sizes on non-adherent conical culture plates (**c**), microtissues with random sizes in gyratory shakers (**d**), and spinner flask (**e**)

contrary, on non-adherent plane culture surfaces, EBs may consist of single-cell clones or EBs that have attached to one another. In the study of tumor biology, microtissues have been produced via spinner flasks (Fig. 1e) or gyratory shakers (Fig. 1d). The constant stirring of the medium in the flask and the flow in the vessel on a gyratory shaker prevent tumor cell lines, e.g., from hepatoma, from attaching to surfaces [13, 21]. The agglomeration of cells on non-adherent concave or conical culture surfaces either uses available commercial non-adherent 96-well plates or are produced by preparation of 96-well plates with agarose medium (Fig. 1c) [7, 22–25]. The agarose medium technique uses the capillary action of the liquid, which creates a concave surface when it becomes solid. According to Hildebrandt and colleagues [24], the most effective and convenient technique for generating microtissues from human bone marrow, compared to the rotation culture and hanging drop technique, is the cultivation of cell suspensions in non-adherent 96-well plates. Non-adherent 96-well plates offered best spheroid formation efficiencies, and the size was best controllable in dependency of the cell numbers seeded per well. For further information on the advantages and disadvantages of different spheroid culture techniques, including aggregate formation efficiency, homogeneity of the aggregates and their viability please refer to the work of Hildebrandt and colleagues [24].

Different cell–cell and cell–extracellular matrix contacts in monolayer cultures and 3D cultures lead to diverse cellular behavior

As already described by Handschel and colleagues in their review of micromass technology in 2007 [26], the basic principle and the advantages of multicellular spheres are the contact of cells between one another and to the extracellular matrix (ECM). In contrast to monolayer cultures where cells are only connected to neighboring cells in two dimensions, a 3D interaction between cells is present in microtissues. This results in differences between localizations as well as the numbers of cell-to-cell contacts, resulting in altered cellular responsiveness and gene transcription profiles [6]. Cukierman et al. provided evidence that even terminal differentiated cells like fibroblasts have higher proliferation rates than cells in monolayer cultures [27]. Moreover, cells are able to change their shape and behavior upon specific cell signals only when they are cultured in 3D, as demonstrated by Weaver et al. for human breast cancer cells [28]. Compared to monolayer cultures, the gene expression profile of liver cells in 3D is much closer to the expression of cells in vivo [29]. In a review of Zhang et al. [5], the importance of the ECM in the development of scaffold materials was highlighted. By referring to the work of Stevens and George [30], they explained that “an ideal scaffold for bone regeneration should be designed based on the constituents and micro- and macrostructure of the native ECM.”

According to Kelm and Fussenegger [31], advances in microtissue production have highlighted the potential of scaffold-free cell aggregates in maintaining tissue-specific functionality, supporting seamless integration of implants into host tissues. In microtissues, cells are connected to ECM proteins in all dimensions, whereas in 2D cultures, the cells only have contact with ECM proteins that are deposited between the culture vessel and themselves. The ECM consists of proteins such as collagens, elastin, and laminin and has several functions in tissues and organs. Among tissue-specific mechanical properties and the transduction of mechanical forces, the ECM influences cellular functions while being simultaneously influenced by the cells [6]. ECM proteins exert their function on cells by interacting with integrins on the cell surface. These receptors specifically bind to motifs located on ECM proteins, i.e., the amino acid sequence RGD of fibronectin [32] and the sequence GFOGER of many collagens [33–35]. Upon binding of cellular integrins, a signaling feedback pathway initiates integrin receptor clustering at the plasma membrane and focal adhesion-associated protein recruitment in osteoblasts [35, 36]. Biggs and Dalby discussed that focal adhesions emerge as diverse protein networks that provide structural integrity and dynamically link the ECM to the intracellular actin cytoskeleton, directly facilitating cell migration and spreading through continuous regulation and dynamic reinforcement [35].

Growth factors and culture supplements that induce or facilitate osteogenic differentiation

The most frequently used method for the osteogenic differentiation of stem cells is incubation with a combination of dexamethasone, ascorbic acid, and β-glycerophosphate (DAG). It seems that dexamethasone induces osteogenic differentiation by upregulating the beta catenin-like molecule TAZ (transcriptional co-activator with PDZ-binding motif), which interacts with the master osteogenic transcription factor Runx2 [37–39]. Moreover, dexamethasone modulates Runx2 activity by upregulating the mitogen-activated protein kinase (MAPK) phosphatase (MKP-1), which leads to the de-phosphorylation of a specific serine of Runx2 and enhanced Runx2 trans-activation [40]. Ascorbic acid contributes to osteogenic differentiation through its role as a co-factor in the hydroxylation of pro-collagen, which then forms collagen that is secreted into the ECM. Collagen is a major protein of the bone matrix [41] and promotes osteogenic differentiation of stem cells [42, 43]. The phosphate of

β-glycerophosphate has two important functions in osteogenesis. First, it is incorporated into the bone mineral hydroxylapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, and second, it phosphorylates extracellular signal-related kinase (ERK) which leads to the expression of many osteogenic genes [44, 45].

In addition to the classical stimulation with DAG, combinations of several growth factors with each other or with DAG have been shown to facilitate osteogenic differentiation in vitro and in vivo. Factors that have a positive effect on osteogenic differentiation include vitamin D3 (vitD3), bone morphogenetic proteins (BMPs), and cyclic AMP (cAMP). BMPs induce osteogenic differentiation via binding to their cell surface receptor, resulting in the phosphorylation and complexation of several SMAD molecules, which then translocate into the nucleus where they induce gene expression and activate Runx2 [39, 46]. Furthermore, BMPs utilize other signaling cascades such as the MAPK cascades and the phosphatidyl-inositol 3-kinase pathway [47]. Fibroblast growth factor is a potent factor for the enhancement of osteogenic differentiation. It acts via inactivation of insulin-like growth factor 1 and transforming growth factor-beta signaling, resulting in enhanced differentiation of mesenchymal stem cells (MSCs), and by an activating phosphorylation of Runx2 after ERK1/2 phosphorylation [48]. Vitamin D3 acts via binding to its vitD3-responsive element in osteogenic genes such as osteocalcin [49]. cAMP induces osteogenic differentiation by binding to the cAMP response element-binding protein which then promotes the expression of the BMP target genes ID-2 and ID-4, resulting in an autonomous stimulation of osteogenesis and a paracrine signaling of BMP2 [50].

Enhanced osteogenic differentiation of microtissues compared to monolayers and probable reasons for this difference

Compared to monolayer cultures, there is an accelerated osteogenic differentiation of cells in microtissues. Whereas mineralized bone nodule formation is first detected after 1 week [51, 52] to 2 weeks [53] in monolayer cultures, bone nodules are already present after 3 days in spheroid cultures (Fig. 2c) [11, 23, 54, 55]. Prior to the formation of bone nodules (see arrows in Fig. 2), multipotent stem cells or osteoblast precursors undergo a complex differentiation process, in which the cells change their architecture from a fibroblastoid to a cuboidal shape and start to produce a bone-like ECM. The bone ECM is mainly composed of collagen type I and several bone-specific proteins, which are a prerequisite for the initiation of mineralization. Specific bone matrix proteins like osteopontin are primary nucleators for the mineralization [56]. Thus, one probable reason for the accelerated mineralization is the enhanced secretion of bone-specific ECM.

Wang and colleagues provided insight into the molecular regulation processes of commercial MSCs (Cambrex, USA) during osteogenesis in spheroids [57]. They demonstrated that markers maintaining the stem cellness were downregulated and that the osteogenic transcription factor Runx2 was upregulated in spherical microtissues [57]. From these findings, they concluded that it was probable that 3D microtissue cultures affected the cell condition, which became sensitive to switching into another cell lineage, resulting in increased osteogenic differentiation. Even more interesting than the enhanced osteogenic differentiation of microtissues in osteogenic medium is the spontaneous differentiation of microtissues from human unrestricted somatic stem cells (USSC) of cord blood [54]. It was demonstrated that the first mineral nodules were present after 5 days in microtissues in normal growth medium [54] (Fig. 2b1). Lammers and colleagues compared the mineralization in microtissues from USSC that were incubated in osteogenic medium with microtissues incubated in standard growth medium with histological staining, scanning electron microscopy, quantitative wavelength-dispersive X-ray spectroscopy (WDX), transmission electron microscopy (TEM), selected area electron diffraction, and Raman spectroscopy [55]. Analysis of the samples with WDX enables for the detection of the element composition of a sample. This analysis showed that mineral nodules of DAG microtissues and control microtissues mainly consisted of calcium phosphate and oxygen. Moreover, the calculated calcium phosphate ratios for both groups were slightly lower (Ca/P 1.52–1.62) than that of HA (Ca/P 1.67). One explanation that was provided was the substitution of Ca by magnesium in the mineral. Furthermore, a substitution of PO_4^{3-} by HPO_4^{2-} in the crystal structure of the mineral would result in a positive charge of the molecule which would be compensated by leaving out a Ca ion. Indications for such a substitution, which results in a so-called calcium-deficient HA, were provided by Raman spectroscopy. In Raman spectroscopy, the oscillation of the P–O binding of the PO_4^{3-} group of calcium phosphate results in a characteristic peak at 960 cm^{-1}. Shoulders and broadening of this peak indicated phosphate groups in which the length of the P–O bonding was affected, such as HPO_4^{2-}, which provided further explanations for the low Ca/P ratio. From further analysis of the crystal growth pattern with TEM, Lammers et al. identified the mineral composition of the samples. They found that the minerals in both groups had similarities with native bone; however, there were differences in the composition of the diverse calcium–phosphate (Ca/P) minerals in the two groups. It was concluded that the mineral of the control group mainly consisted of calcium-deficient HA (CDHA) with an amorphous mineral fraction (ACP), while the mineral formed in

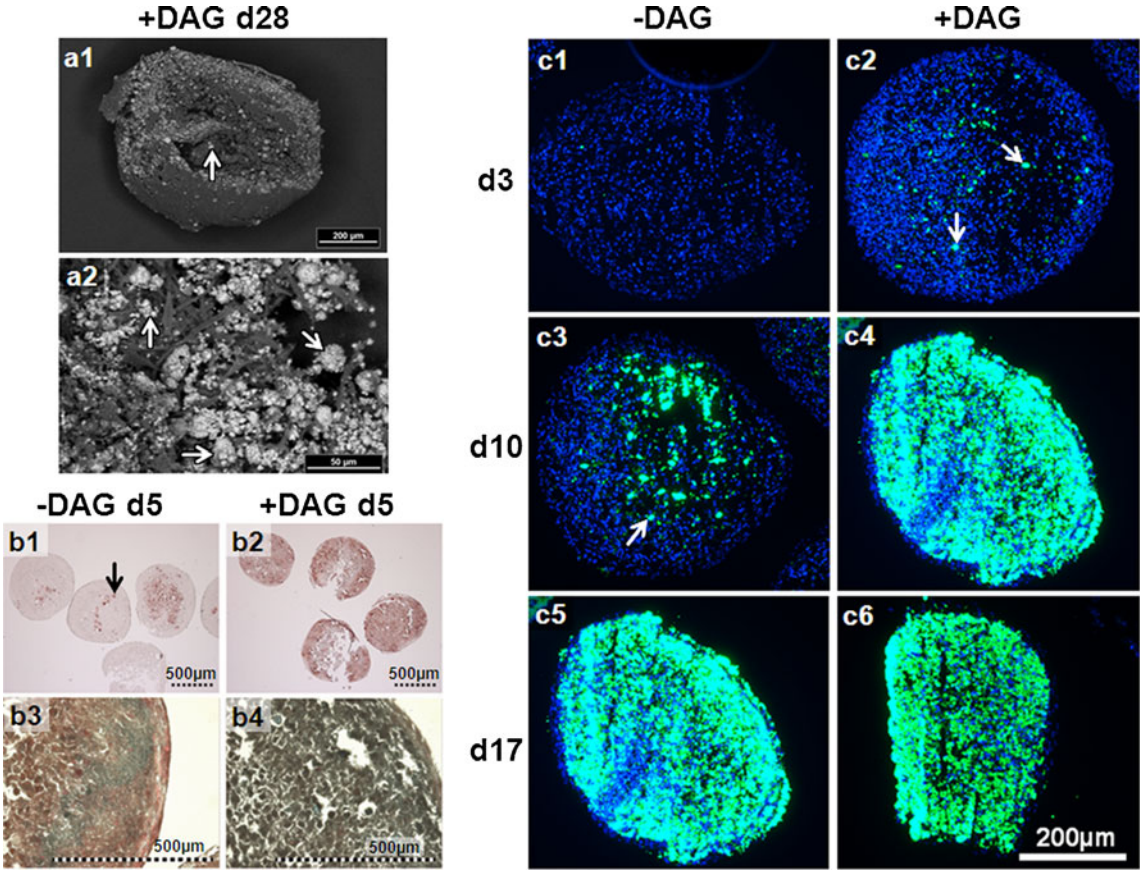

Fig. 2 Mineralization of microtissues after 28 days in osteogenic medium. *a1* Scanning electron microscopic image of a microtissue cross section. *b1* Bone nodules can be detected between the cells of the microtissue (white arrows). Alizarin red S staining (*b1–b2*) and Masson Goldner staining (*b3–b4*) of USSC microspheres, incubated with or without DAG for 5 days. Calcium is stained *dark red* (*b1–b2*); extracellular matrix is *green* and cytoplasm is *red* (*b3–b4*). *b1–b4* A modification of Fig. 2 of Langenbach et al. (http://online.liebertpub.com/doi/abs/10.1089/ten.TEA.2009.0131?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed) [54]. OsteoImage staining of microtissues with or without DAG after 3, 10, and 17 days. *White arrows* in DAG microtissues after 3 days (c2) indicate early mineralization

the osteogenic medium group mainly consisted of ACP, octa-calcium phosphate, magnesium whitlockite, CDHA, and HA. Even though HA is the predominant mineral in bone, several other forms of apatitic minerals are present. As early as in 1987, Legros et al. [58] demonstrated that the mineral of bone samples, regardless of species (rat or bovine) or age, was found to be a calcium-deficient apatite containing both CO_3^{2-} and $HPCO_4^{2-}$ ions in the crystal lattice. Furthermore, they showed that the Ca/P ratio increased with age from 1.51 in newborn rats, which was the same ratio as that in microtissues of Lammers et al. [55], to 1.69 in adults.

Currently, there is no literature about the precise mechanisms that drive this enhanced and spontaneous differentiation. However, there is some evidence showing that contact with ECM proteins plays an essential role. Integrins transduce extracellular signals via a molecule called focal adhesion kinase (FAK), a protein that is constitutively associated with the β-integrin subunit [59]. FAK itself functions as an initiator of multiple signaling cascades. After the activating phosphorylation of FAK, signaling cascades are initiated that regulate the synthesis of osteospecific proteins. How important these mechanisms of ECM protein induced and integrin → FAK-mediated signaling are in the osteogenic differentiation of stem cells and in osteoblasts was explained by Xiao and colleagues: "(1) Osteoblasts must be in contact with a collagen-containing ECM before they can differentiate. (2) Osteoblasts bind to this ECM via interactions between Col1 and α2β1 integrins. (3) Integrin ligand binding activates MAPK and related pathways that transduce signals to the nucleus. (4) Runx2 is phosphorylated and activated by MAPK, thereby allowing it to stimulate osteoblast differentiation by increasing transcription of osteoblast marker genes such as OCN" [42, 60]. A large body of evidence for this theory has been provided by experiments, which have demonstrated that blocking of α2β1 integrins as well as treating cell cultures with collagenase suppresses osteogenic differentiation [61, 62]. The other way round, the

contact of human MSCs with compartments of the ECM (vitronectin and Col1), is sufficient to induce osteogenic differentiation [43, 51] and the expression of osteogenic transcription factors like osteonectin [63]. Moreover, this upregulation is correlated with an increase in the expression of collagen type I. It is suggested that contact with the ECM secreted by the cells themselves leads to the above-mentioned integrin → FAK-mediated activation of osteogenic transcription factors.

Another inducer of osteogenic differentiation processes may be the high cell density inside the microtissues. It is well-known that osteogenic differentiation in vitro requires high cell densities and that osteogenic differentiation is restricted during cell proliferation [64]. This is supported by the findings of Bitar et al., who demonstrated that the expression of the osteogenic transcription factor Runx2 was upregulated as a consequence of higher cell densities [65]. Moreover, Jahn et al. showed that the transformation of the osteoblast phenotype in vitro into a more mature stage could be achieved more rapidly in 3D culture and that dense monolayers elicited more mature osteoblasts than low-density seeded monolayers, while hOB cells in pellets seemed to have transformed even further along the osteoblast lineage [66].

Recently, Kabiri et al. [67] found that autocrine BMP signaling may be responsible for increased osteogenic differentiation of cellular aggregates. They found an approximately 30-fold upregulation in BMP2 expression after 1 week of culture. This increase was not accompanied by increased upregulation of osteonectin, osteopontin, ALP, Runx2, and collagen type I on the same day, but led to an increase in these factors after 14 days of culture when BMP-2 levels had already returned to basal expression levels. Interestingly, the increase in BMP2 expression was not dependent on the osteogenic medium; it also increased 25-fold in control medium compared to 2D cell cultures. These findings provide further evidence that osteogenic differentiation of stem cells is initiated by the contact of cells with one another and to the ECM that surrounds the cells. Phimphilai et al. found that autocrine BMP2 production is necessary for the function of Runx2 and that Runx2 and BMP2 cooperatively interact to stimulate osteoblast gene expression [68]. It has been demonstrated earlier that murine pre-osteoblast cell lines and marrow stromal cells produce basal levels of BMPs that are essential for osteogenic differentiation [69]. Bone marrow aspirates contain an inherent osteogenic cell population, called skeletal stem cells (SSC), that is, with exception to some neo- and prenatal cells (e.g., from cord blood) the only cell type that is able to form bone including a hematopoietic microenvironment [70–72]. As SSCs and presumably also USSC are inherently osteogenic, 3D culture is probably enough to initiate osteogenic differentiation. In this process, the activating phosphorylations of Runx2 by ERK after interaction of the cell with collagens, cell–cell contacts and growth factors in the extracellular space, are sufficient triggers for the differentiation.

Apoptosis of osteoblasts in vivo is an important factor to control the number of osteoblasts that are involved in new bone formation inside bone remodeling units. In this process, some osteoblasts are entombed within the matrix as osteocytes but the majority die by apoptosis [73]. Currently, there are controversial results of whether stem cells undergo apoptosis in microtissues that are incubated under standard medium conditions. Kelm and colleagues [74] demonstrated massive apoptotic processes inside microtissues of mouse, rat, and human MSCs by TUNEL assays and immunohistochemical detection of caspases. Furthermore, Hildebrandt and colleagues [24] found reduced viability per diameter in control microtissues. By contrast, Lammers and colleagues found no apoptosis, but spontaneous osteogenic differentiation of USSCs [55]. Whether these differences are caused by different cell lines or standard culture conditions remains unclear. Whereas Lammers and colleagues used a very high concentration of fetal calve serum (FCS), i.e. 30 %, Kelm only used 10 % FCS [74]. Interestingly, apoptosis was suppressed in cultures of Kelm and colleagues that were exposed to osteogenic medium. This is again supported by Hildebrandt and colleagues [24], who demonstrated relatively constant viability per diameter in treated osteogenic microtissues. Thus, it is probable that there is a strong correlation between the suppression of apoptosis and osteogenic differentiation. Kelm hypothesized that in an environment lacking the appropriate biological cues for maintaining their undifferentiated state, MSCs have to differentiate for sustained survival or otherwise undergo apoptosis [74]. This can be regarded as a "biological safety switch" to prevent adverse effects of MSCs in ectopic organs. From this point of view, it is probable that USSCs, which have a higher multipotent plasticity compared to MSCs from bone marrow, are able to compete with cues provided in microtissues by differentiating into the osteogenic lineage.

Current and probable applications for microtissues in osteogenic tissue engineering

Recently, Altmann et al. concluded that the 3D culture of osteoblasts or MSCs, which are both known to require 3D microenvironments for proper adhesion, growth, aggregation, and/or tissue-specific differentiation, provides a promising tool for in vitro pre-conditioning into a mature osteoblast phenotype for applications in bone augmentation and hard tissue regeneration [75].

In previous works, we and others have demonstrated that microtissues of osteoblasts or USSCs can be osteogenic

differentiated while maintaining the ability to let cells divide and migrate to the surrounding tissue [54, 76]. This characteristic is extremely important when microtissues are implanted in vivo, in case of gaps that need to be colonized by osteogenic cells. It was found that the optimal differentiation time for microtissues is 3 to 5 days in order to maintain outgrowth capability. The cells that migrate out of the microtissues probably derive from the cells of the surface of the microtissues, which migrate and divide. In another study, these results were transferred to a model for the enhanced inoculation and osteogenic differentiation of scaffolds [25]. It was shown that microtissues of USSCs could be implanted into insoluble collagenous bone matrix scaffolds (ICBM) where they mineralized after a short time and allowed cells to migrate to the surrounding scaffold material. In this process, they partially filled spaces between the microtissues and the scaffold material, as well as spaces between adjacent trabeculae of the spongiosa. Compared to inoculation with cell suspensions, a 40-fold higher cell load on a single ICBM is feasible [25].

According to Ferrera et al., subcutaneous implantation in nude mice led to a high rate of success in progression throughout differentiation of implants (12 of 12), independent of donor age and gender (25 to 73 years of age). This offers the possibility of implanting structures at different and controlled stages of osteogenic progression [76]. Furthermore, microtissues allow the precise administration of large numbers of cells into a specific area and have the advantage of strong rigidity that prevents dissociation of the cells inside the tissue [23]. Kelm and Fussenegger stated that microtissues are not as susceptible to wash out as single cells due to their larger size and are significantly more adhesive than monolayer cultures or single cells because of increased ECM production [77, 78]. This characteristic is of particular importance in approaches that combine implant materials with stem cells that have not been preincubated with a scaffold. Multicellular complexes that reside at the implantation site could improve the stability of the implant-to-tissue contact, in contrast to single cells that are applied as suspensions. Moreover, there is a potential use of microtissues combined with membranes in the healing of periodontal defects. According to Berahim and colleagues, a periodontal defect could be filled by migrating cells that derive from the division of cells in microtissues that were previously seeded on membranes [79]. The washing away of the spheres and the cells by crevicular fluid could be prevented combining of membrane-facilitated guided tissue regeneration with microtissues. Finally, the suitability of microtissues for osteogenic tissue engineering was demonstrated in a previous work from our group. ICBM scaffolds implanted with microtissues were shown to mediate ectopic bone formation upon implantation in rat muscle bags, whereas scaffolds implanted without cells did not lead to bone formation [22].

To conclude, microtissue technology provides better in vivo-like conditions for stem cells and osteoblasts than monolayer cultures, accompanied by improved osteogenic differentiation. Microtissue technology can improve seeding efficiency of biomaterials by increasing the cell load of a scaffold and are less prone to wash out than single cells. This results in accelerated osteogenic tissue formation and therefore could contribute to earlier implant stability in mandibular bone augmentation. Moreover, microspheres hold promise for facilitated, accelerated, and improved generation of tissue-engineered bone and stem cells to support the healing of periodontal defects.

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Conflict of interest The authors declare that they have no conflict of interest.

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