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Growth Factors and Signalling Molecules for Cartilage Tissue Engineering – from Embryology to innovative release strategies for Guided Tissue Engineering

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1. Introduction

Modern Tissue Engineering represents a highly complex interdisciplinary branch of science which brings the life sciences and engineering together to design innovative solutions for the treatment of critical tissue defects after traumatic or degenerative damage. In this area an interesting evolution can be observed in the approach to achieve this goal: Originally understood as a principally biomaterial-based strategy using natural and synthetic materials as implants to be integrated into the neighbouring tissue, during the last two decades a change of paradigm has taken place which involves a movement away from the structural replacement of damaged tissue towards strategies to regenerate functional tissue (Kirkpatrick et al., 2006). In this approach cells play an important role, for example in seeding scaffold materials, which give a preformed structure for the regenerating tissue. A major aim is the synthesis of so-called tissue-like structures *in vitro* which can be implanted in the damaged tissue site and should be integrated into the body's own tissue. Relevant obstacles to this strategy are the proper morphological integration of the cell-material construct in the structural context of the damaged tissue site as well as the maintenance of its function. The use of cells in combination with different biomaterials gives an important

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input for the further development of innovative materials with the important aims of preserving cell viability, improving the cell seeding efficiency of a scaffold material and by this to enhance their functionality.

Due to the limited regenerative potential of cartilage, traumatic damage to articular cartilage is a unique example for the clinical relevance of tissue engineering strategies to treat damaged tissue. In this context, the establishment of the autologous chondrocyte transplantation by Brittberg et al. (1994) represents the first innovative step to treat articular cartilage damage via tissue engineering. With the help of a syringe a suspension of *ex vivo* expanded chondrocytes was injected into the defect which had been covered with a periosteal flap. The current approach involves the expansion and transplantation of the autologous chondrocytes with the help of a matrix. For this purpose different material-based matrices are already on the market and have been developed to provide the seeded cells with a suitable biomimetic microenvironment.

Modern tissue engineering approaches integrate the combined use of cells, scaffolds, and growth factors or signalling molecules respectively. There is the hope that such molecules will stimulate the controlled proliferation and proper differentiation of the seeded cells in the scaffold. Furthermore, growth factors should also enhance the migration, proliferation, and differentiation of cells from the edges of the treated defect. Thus, such factors can serve first to optimize tissue engineered cell-scaffold constructs *in vitro*, and second to improve their performance and integration *in vivo*. For the targeting of potential signalling molecules and growth factors it is important to realize that regeneration in part recapitulates developmental processes. Therefore, it can be postulated that for the improvement of regeneration in the context of tissue engineering it is essential to understand the regulation of developmental processes (Reddi, 2003). With this purpose in mind our group has demonstrated that the growth plate of the long bones represents a suitable developmental model to target interesting molecules for tissue engineering application of cartilage and bone (Brochhausen et al., 2009).

In this contribution we firstly focused on the structural organization and regulation of the growth plate. Based on this we present the development of an integrated release-system in an oriented gelatine-based scaffold material for cartilage tissue engineering.

2. Long bone development and endochondral ossification

Two different steps in bone development are distinguishable, both being important for potential tissue engineering strategies: first, the condensation of mesenchymal cells as a morphological template and its development to a cartilaginous template during fetal development, and second, the longitudinal growth of the long bones within the area of endochondral ossification (fig. 1). In the present report we very briefly present the process of foetal bone development and then concentrate on endochondral ossification and its regulation.

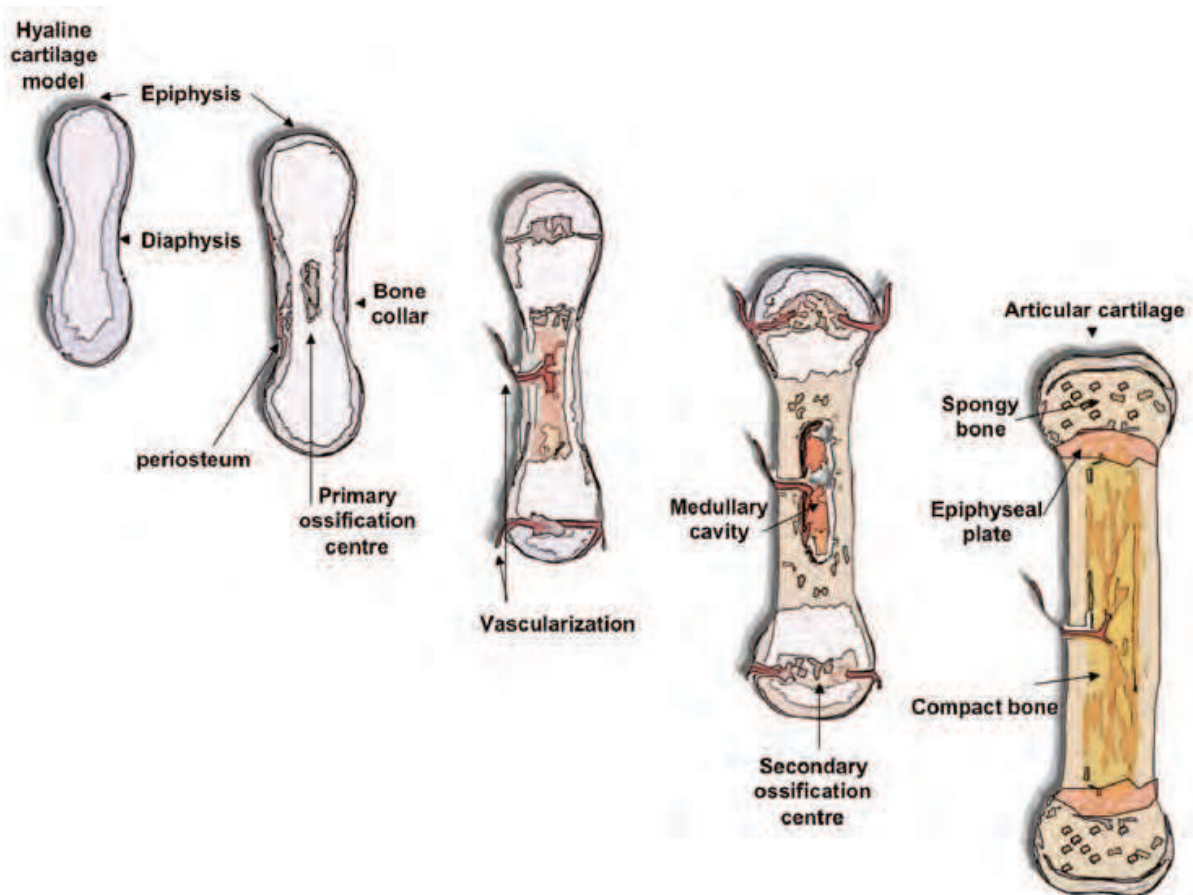


Fig. 1. Schematic depiction of bone development from the hyaline cartilage model to the ossified bone with the growth plate as the epicentre of the long bone growth.

The growth plate of long bones represents the epicentre of the long bone longitudinal growth. In this structurally highly organized region between the epiphysis and the metaphysis of the bone a cartilaginous template is responsible for longitudinal growth, which results in terminal ossification and thus leads to the elongation of the long bone (fig.2). The characteristic features of this region are chondrocytes in different proliferation and differentiation stages integrated in a scaffold of oriented extracellular matrix components, mainly consisting of different types of collagen. Since proliferation and differentiation in this region is a well regulated process the growth plate can be divided into characteristic horizontal zones. In these zones not only the chondrocytes showed a specific phenotype, but also specific types of collagen can be found within the different zones.

2.1 The resting zone

The zone closest to the epiphyseal cartilage is the smallest zone, called the resting zone. The latter consists of monomorphic, small chondrocytes with large central nuclei and a narrow cytoplasmic rim. Nowadays, the resting zone cells are seen as being the cell pool for the adjacent proliferative zone, since they contain so called "stem-like" cells to supply new clones of proliferative zone chondrocytes. This hypothesis could be confirmed by findings of Abad et al. (2002), who demonstrated that, after removing the proliferative and the

hypertrophic zones of the growth plate, a complete proliferative and hypertrophic zone was regenerated, based on cells from the residual resting zone alone. Furthermore, the chondrocytes of the resting zone might produce a morphogen, so-called Growth Plate-Orienting Factor (GPOF) that directs the alignment of proliferative clones into columns parallel to the long axis of the developing bone. The GPOF diffuses into the proliferative zone along a concentration gradient. This gradient is thought to guide the orientation of the proliferative chondrocytes.

The stem-like cells in the resting zone have a defined proliferative capacity, which is gradually exhausted and leads to fusion of the growth plate. Thus, the epiphyseal fusion process seems to be the result and not the cause of growth cessation at the moment of skeletal maturation. Accordingly, the term "growth plate senescence" has been introduced by Nilsson and Baron (2004). In this context, Stevens et al. (1999) demonstrated in transplantation experiments the dependence of the growth rate on the age of the donor animal, not that of the recipient. This finding indicates that the programmed senescence is not caused by hormonal or other systemic mechanisms but is intrinsic to growth plate cells themselves. In further investigations Gafni et al. (2001) showed that senescence is a function of proliferation: After systemic treatment with dexamethasone, a glucocorticoid that slows chondrocyte proliferation, the treated growth plates showed a delay in senescent decline of the growth rate, and the epiphyseal fusion occurred later.

2.2 The proliferative zone

After entering the cell cycle the cells begin to proliferate. During mitosis the cells divide in the horizontal axis followed by a rotation and finally the arrangement in the vertical axis forming the characteristic columns parallel to the longitudinal axis of the bone (Aszodi et al., 2003). These columns are separated from each other by matrices with large amounts of collagen Type II. Each column contains 10 to 20 cells, depending on the mitotic activity of the chondrocytes. In the proximal portion of each column, multiple mitotic figures can be observed. It is interesting that the achromatic figure spindles are arranged parallel to the long axis of the bone and that the chondrocytes migrate after division so that eventually the daughter cells are lined in the longitudinal axis (Trueta & Morgan, 1960; Stevenson et al., 1990). The proliferating zone is the only region in the growth plate where chondrocytes have relevant mitotic activity. Biochemically, in this zone the high oxygen content is noteworthy, as well as the high glycogen content and increased mitochondrial ATP production in chondrocytes (Brighton & Heppenstall, 1971).

2.3 The hypertrophic zone

After 5-10 mitotic cycles the chondrocytes lose their proliferative capacity and enter the so-called G0 phase for further differentiation. After passing a prehypertrophic stage chondrocytes enter a hypertrophic differentiation stage, which in the growth plate unidirectionally leads to terminal differentiation. During this process the chondrocytes increase their volume, especially in their longitudinal diameter, about 5- to 10-fold. In this way, hypertrophic differentiation makes an important contribution to longitudinal growth. Furthermore, the hypertrophic cells synthesize high amounts of extracellular matrix components, especially collagen Type X (Hunziker et al., 1987; Breur et al., 1991). In comparison to the resting zone and the proliferative zone, the hypertrophic zone is

characterized by a distinctly different extracellular matrix composition: The synthesis of the collagens Type II, IX and XI is dramatically reduced, whereas the production of collagen Type X increases. In fact, collagen Type X is only expressed in this zone of the growth plate and hence, it is considered to be a specific marker for chondrocytic hypertrophy. Furthermore, hypertrophic chondrocytes show a significant increase of intracellular calcium concentration. Intracellular calcium is essential for the production of release vesicles (Wang & Kirsch, 2002), which are rich in several types of Ca²⁺-binding annexins which promote Ca²⁺ uptake by these vesicles (Kirsch et al., 2000; Wang et al., 2003). Calcium phosphate, hydroxyapatite, phosphatases such as alkaline phosphatase, and metalloproteinases are secreted via exocytosis, thus driving proteolytic remodelling and mineralization of the surrounding matrix (Lewinson & Silbermann, 1992; Vu et al., 1998). Moreover, the hypertrophic chondrocyte is a crucial master regulatory cell with an active metabolism. It prepares the matrix for calcification and vascularization (Forriol & Shapiro, 2005). In this context the expression of Vascular Endothelial Growth Factor (VEGF) by hypertrophic chondrocytes is of special interest for the initiation of the terminal ossification process.

2.4 The invasive zone

The expression of VEGF attracts invading blood vessels and, concomitantly, osteoblastic cells (Gerber et al., 1999). After vascularization of the mineralized cartilaginous matrix, the matrix is degraded by immigrant chondroclasts/ osteoclasts (Lewinson & Silbermann, 1992; Vu et al., 1998). The main part of the longitudinal septa (up to 80%) within the hypertrophic cartilage is rapidly resorbed behind the invading blood vessels, whereas the remaining septa serve as scaffolds on which osteoblasts deposit bone matrix (Salle et al., 2002). Finally, triggered by caspases, the hypertrophic chondrocytes undergo apoptosis or are resorbed (Gibson, 1998; Salvesen & Dixit, 1997).

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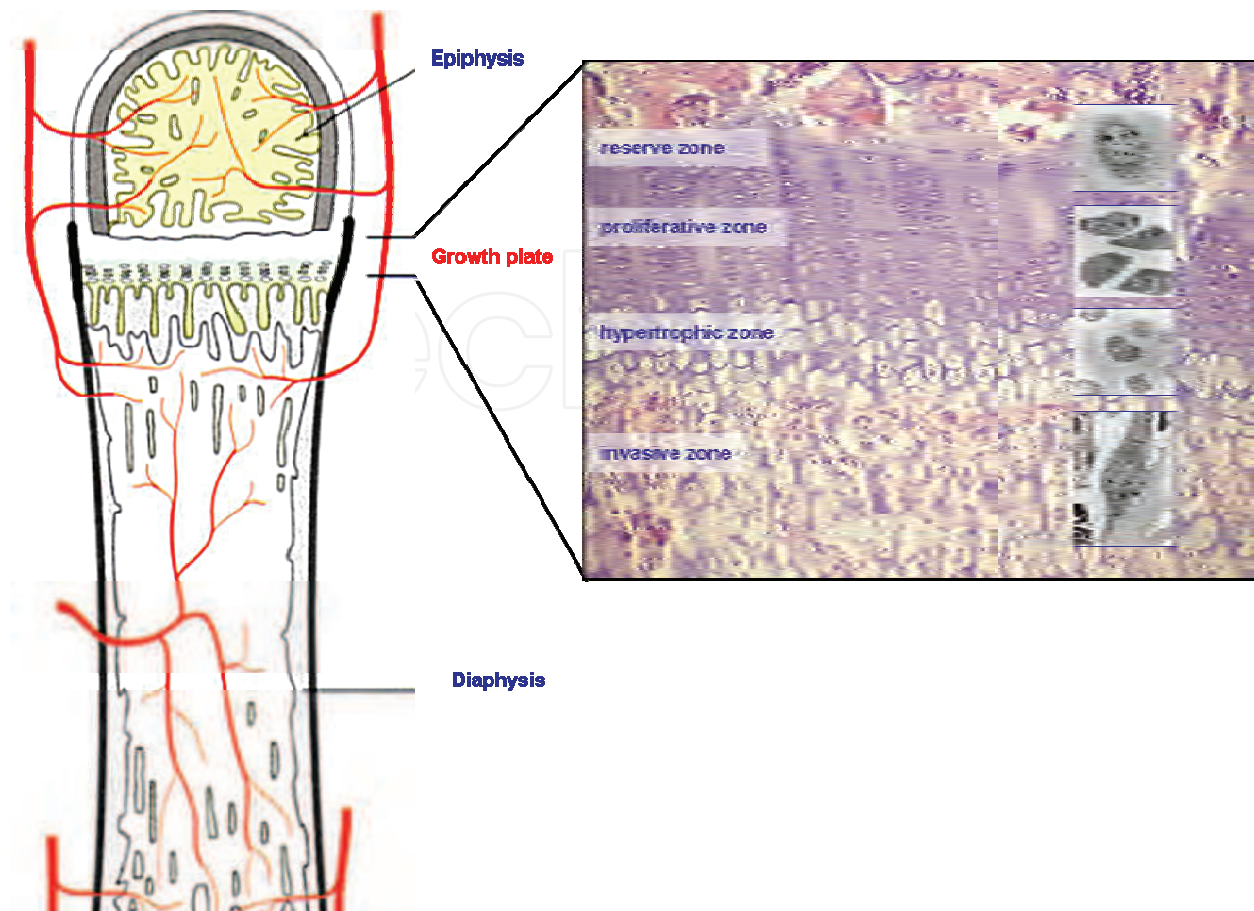


Fig. 2. The growth palte is a well organized structure between the epiphysis and the diaphysis of the long bones. The chondrocytes are arranged according to there proliferation and differentiation stage with small round shaped cells in the resting zone, flat, proliferating cells in the proliferative zoe and great cells in the hypertrophic zone. In the invasive zone vessels grow in and give rout for the final ossification (HE and transmission electron microscopical images).

3. Molecular Regulation of Endochondral Ossification

The complex interplay between chondrocyte proliferation and differentiation, which is responsible for the characteristic morphological features of the growth plate, is highly orchestrated by the interaction of multiple systemic hormones and paracrine factors, which finally lead to changes in gene expression of the chondrocytes in the growth plate. The fundamental importance of endochondral ossification in long bone growth and development as well as in repair mechanisms of the skeleton is one reason for the increasing interest in the molecular factors that regulate this critical pathway. This section describes the latest insights into the regulation of chondrocyte proliferation and differentiation within the growth plate and focusses on molecules that play key roles in embryology and development. The description represents a choice of some important molecules without any claim to be exhaustive. A more precise review of the different pathways involved in growth plate with respect to its potential relevance for tissue engineering is given elsewhere

(Brochhausen et al. 2009). Table 1 summarizes the most important characteristics and effects of the signalling molecules and growth factors, which are described in detail below.

3.1 Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) represent a group of well known growth factors of the Transforming Growth Factor superfamily (TGF-beta). They were first identified and described by their capacity to stimulate ectopic cartilage and bone formation (Wozney, 1989). On this basis, BMP's represent a prime example of the high potential of growth factors for tissue engineering strategies. Today their important functional role in osteogenesis and chondrogenesis is well established. In early fetal development BMPs play important roles in the aggregation of mesenchymal cells during limb bud formation (Pizette & Niswander, 2000). In addition, BMPs influence the complex mechanisms in developing bone at later stages of skeletal development. The cellular effects of BMPs are mediated by two type I receptors, namely BMPRIA and BMPRIB, which heterodimerize with a type II receptor (BMPRII). The expression of BMPRIB was described by Zou et al. (1997) in early embryological development during mesenchymal cell condensations, which gives evidence that this receptor is involved in early bone and cartilage formation. The receptor BMPRIA could be detected in prehypertrophic chondrocytes (Zou et al., 1997). The differential expression of these receptors indicates the involvement of BMP-related pathways in different steps of bone and cartilage development. In this context it is of interest that BMPs have different expression patterns within bone and cartilage: BMP2, -3, -4, -5, and -7 are expressed in the perichondrium. BMP2 and -6 are expressed in hypertrophic chondrocytes and BMP7 is expressed in proliferating chondrocytes.

BMPs are integrated in the Ihh-PTHrP (Indian hedgehog-parathyroid hormone-related peptide) signalling loop. They are capable of inducing the expression of Ihh and, hence, increase chondrocyte proliferation (Grimsrud et al., 2001). Furthermore, BMP pathways inhibit FGF signalling by constraining the expression of FGFR1 (fibroblast growth factor receptor 1) (Yoon et al., 2006). Within the growth plate a concentration gradient of BMP may be a key feature responsible for spatial regulation of chondrocyte proliferation and differentiation (Nilsson et al., 2007).

Regarding tissue engineering strategies it is not surprising that recombinant human BMP's have already found their way into clinical practice for the treatment of critical sized bone defects. To further optimize the use of these factors in tissue engineering the identification and synthesis of well-defined carriers with suitable BMP release profiles is necessary as described by Bessa et al. (2008). A detailed description of the physiological function of the different BMP's during bone development is given by Kronenberg (2003), Forriol and Shapiro (2005).

3.2 Fibroblast Growth Factor-signalling

Fibroblast Growth Factors (FGFs) represent a superfamily of peptide growth factors synthesized by a variety of human and animal cells and act in a paracrine manner on their target cells. The cellular effects are mediated by plasma membrane tyrosine kinase receptors. The 22 distinct human FGFs are involved in diverse biological processes. In this context, a temporal selectivity in their expression is involved in the regulation of these pathways: the expression of FGF3, FGF4, FGF8 and FGF17 is restricted to embryonic tissue, whereas other

FGFs play significant roles not only in developmental processes but also in the biology of mature tissues (Jones, 2008). A further discrimination of the effects in the FGF-related pathways is given by the mediation via four different types of FGF receptors (FGFR).

Regarding skeletal development FGF1, 2, 17, and 19 are the predominant FGF members present in developing human cartilage (Krejci et al., 2007). In the early stage of endochondral bone formation multiple FGF's as well as FGFR2 are expressed in condensing mesenchymal cells, which is thought to stimulate SOX9 expression (Murakami et al., 2000). FGFR3 is expressed by proliferating chondrocytes in the human growth plate, whereas FGFR1 is located in prehypertrophic and hypertrophic chondrocytes. Additionally, FGFR2 can be detected on perichondral cells. Each of these receptors has distinct roles in bone development. At the moment FGFR3 is best understood: FGFR3 inhibits the proliferation of growth plate chondrocytes, especially by down-regulating Ihh expression in hypertrophic chondrocytes. Hence, FGF signalling shortens the proliferative columns. Furthermore, FGFs decelerate chondrocytic terminal differentiation. Thus, they decrease matrix synthesis and consequently delay hypertrophy of the growth plate chondrocytes (Kato & Iwamoto, 1990). FGF18 is expressed in the perichondrium and by an activation of FGFR3 this factor is thought to regulate skeletal vascularization (Liu et al., 2007). The crucial role of FGFR3-mediated pathways in skeletal development is illustrated by the fact that a specific mutation in FGFR3 causes achondroplasia, the most common dwarfism in humans (Bellus et al., 1995; Shiang et al., 1994). A comprehensive overview of FGF and its functions during skeletal development is given by Olsen et al. (2000), Kronenberg (2003) as well as by Forriol and Shapiro (2005).

3.3 Parathyroid Hormone-related Peptide (PTHrP) and Indian hedgehog (Ihh)

PTHrP is a peptide which shares a receptor with the parathyroid hormone and has at its amino-terminus the identical 13 aminoacids as in the hormone itself. However, in contrast to the hormone PTHrP is detectable in many adult and developing tissues (Ingleton and Danks, 1996). Functionally, PTHrP is much more related to developmental growth factors such as FGF or TGF-beta than to a hormone. The identification of the PTHrP/Ihh feedback loop led to a major advancement in our understanding of growth plate regulation since it became clear that this pathway regulates the entry of chondrocytes into the hypertrophic phase of growth (Alvarez et al. 2003). The interplay between PTHrP and Ihh determines the moment when the chondrocytes leave the proliferative zone and enter the transition to hypertrophic chondrocytes. Perichondral cells and chondrocytes in the periarticular perichondrium synthesize PTHrP. This protein diffuses toward the prehypertrophic zone where cells have a high expression level of PTH/PTHrP receptors. After binding, PTHrP inhibits further differentiation to hypertrophic chondrocytes and keeps the chondrocytes in the proliferative stage (Lee et al., 1996). In the moment when the diffusion distances from the PTHrP-producing cells become too long, the effector cells are no longer sufficiently stimulated by PTHrP, which results in a delay of proliferation and the synthesis of Ihh, a member of the hedgehog family. Indian hedgehog is not only important in embryological development but also in the regulation of the growth plate. In this context Ihh is synthesized by prehypertrophic chondrocytes. As a consequence, the rate of chondrocyte proliferation increases and, by mechanisms that are still not fully understood, Ihh stimulates the production of PTHrP at the bone ends. Furthermore, by action of Ihh perichondral cells convert into osteoblasts and thus, enhance the formation of the bone collar. The essential

role of *Ihh* in the postnatal integrity of the growth plate and endochondral ossification was clearly demonstrated by Maeda et al. (2007).

3.4 Vascular Endothelial Growth Factor

In the final ossification of the growth plate which leads to the formation of new bone the vascularisation of hypertrophic cartilage represents a crucial step. Thus, it is important that hypertrophic chondrocytes of the growth plate produce and secrete Vascular Endothelial Growth Factor (VEGF), one of the most potent angiogenic factors (Gerber et al., 1999). VEGF in turn stimulates the proliferation and migration of endothelial cells. It enhances the formation of blood vessels in the hypertrophic zone of the growth plate and with this paves the way for ingrowth of osteoblasts, thus leading to ossification. Furthermore, the vascularization of the calcified cartilage within the invasive zone is essential for the resorption of chondrocytes by chondroclasts. Experimental data were able to demonstrate that the absence of VEGF causes profound disturbances in the architecture of the growth plate and affects significantly the longitudinal growth. In the absence of VEGF calcified cartilage persists as a result of a decrease in the recruitment and differentiation of osteoblasts and /or chondroclasts. As a morphological consequence a widening of the hypertrophic region and diminished trabecular bone formation could be observed as a result of the reduced elimination of terminally differentiated chondrocytes (Gerber et al., 1999). Thus, VEGF-mediated capillary invasion is an essential signal that triggers cartilage remodelling and hence influences growth plate morphogenesis. Interestingly, hypertrophic chondrocytes also express receptors for VEGF, which leads to the hypothesis that this factor could have an autocrine function in these cells (Carlevaro et al., 2000). In addition to VEGF, ligands for the tyrosine kinase receptor Tie2 are further key regulators of angiogenesis and post-angiogenic blood vessel maturation (Asahara T, 1998, Suri C et al., 1996). The investigations of Horner et al. (2001) revealed the expression and spatial distribution of angiopoietin-1 and -2 (Ang-1 and Ang-2), the ligands for the endothelial receptor Tie-2 in the human growth plate. This group described Ang-1 and Ang-2 as an important regulator in the orchestration of neovascularization in the growth plate. In this context, Ang-1 and Ang-2, which were expressed by hypertrophic chondrocytes of the growth plate, seemed to modulate the effects of VEGF (Horner et al., 2001). The fundamental role of VEGF during long bone development is reviewed in detail by Ballock and O'Keefe (2003).

3.5 Sox transcription factors

Sox proteins are a member of the High-Mobility-Group (HMG) superfamily, which represents DNA-binding proteins, whose sequence is at least 50% identical with the sequence of the HMG domain of the sex-determining region located on the Y chromosome of human and other mammals (Sinclair et al., 1990). This relation is responsible for the name of the Sox protein family (Wegner, 1999). During long bone development Sox9 is critically involved in several stages during the developmental processes. In early embryological development Sox9 is required for the aggregation of mesenchymal cells (Bi et al., 1999). Sox5 and Sox6, two other members of the Sox family, are coexpressed with Sox9 in chondroprogenitor cells and there is strong evidence that these genes are clearly needed for the differentiation of the chondrocytic lineage (Lefebvre et al., 1998; Akiyama et al., 2002). Furthermore, Sox9 is necessary for the expression of various extracellular matrix

components, especially the collagen Types II, IX, XI as well as aggrecan (Bi et al., 1999; Lefebvre and de Crombrughe, 1998). At later stages of development, the presence of Sox9, partly mediated by Sox5 and Sox6, is essential for chondrocyte proliferation and the alignment of proliferative clones into columns parallel to the longitudinal axis of the developing bone, indicating their important role in the proliferative zone of the growth plate (Akiyama et al., 2002). This fact is further illustrated by the fact that the expression of the Sox5, Sox6, and Sox9 genes is completely turned off in hypertrophic chondrocytes (Lefebvre et al., 1998). Functionally, in the proliferative zone of the growth plate Sox9 prevents the transition of proliferating chondrocytes into hypertrophic chondrocytes, and is thus involved in the control of subsequent endochondral ossification (Akiyama et al., 2002). Furthermore, Zehentner et al. (1999) provided evidence that BMPs are also modulated by the expression of Sox9. With the help of transducing experiments Hardingham et al. (2006) demonstrated the critical role of Sox9 in chondrogenesis of isolated human articular chondrocytes. Regarding possible tissue engineering strategies for cartilage, Khan et al. (2009) concluded that chondroprogenitors maintain SOX9 expression during extended monolayer culture and further retain chondrogenic potential. These findings demonstrate that the sox transcription factors not only play a crucial role in endochondral ossification but also in establishing the phenotype of articular cartilage both *in vivo* and *in vitro*, indicating that the regulation of the growth plate represents a suitable model to target potential signalling molecules and growth factors for cartilage tissue engineering.

3.6 Runx2

Runx2 (also known as Cbfa1) is another important transcription factor of the Runt-domain family (Ito, 1999). Runx2 is expressed during condensation of mesenchymal cells in early bone development and later in osteoblasts, but also in hypertrophic chondrocytes (Ducy et al., 1997; Inada et al., 1999; Kim et al., 1999; Otto et al., 1997). The analyses of Runx2 deficient mice gave evidence for the crucial functions of Runx2 during skeletal development: This transcription factor is firstly required for the differentiation of mesenchymal progenitor cells into osteoblasts. Later on in the growth plate of the long bones Runx2 enhances chondrocyte terminal differentiation (Inada et al., 1999; Kim et al., 1999) opening up the way for subsequent ossification. In Runx2-deficient mice, no endochondral and no intramembranous bone formation is present due to an arrest in osteoblast differentiation (Otto et al., 1997; Komori et al., 1997). In such mice, most bones either lack, or have reduced numbers of hypertrophic chondrocytes. In addition, hypertrophic chondrocytes fail to mineralize their matrix and have reduced or undetectable expression levels of osteotypical genes, such as osteopontin and MMP-13 (Takeda et al., 2001; Ueta et al., 2001). Thus, Runx2 is required for the differentiation of mesenchymal progenitor cells into osteoblasts. Furthermore, in the growth plate of long bones, this transcription factor enhances chondrocyte terminal differentiation (Inada et al., 1999; Kim et al., 1999) and is therefore a crucial factor for the definitive formation of trabecular bone.

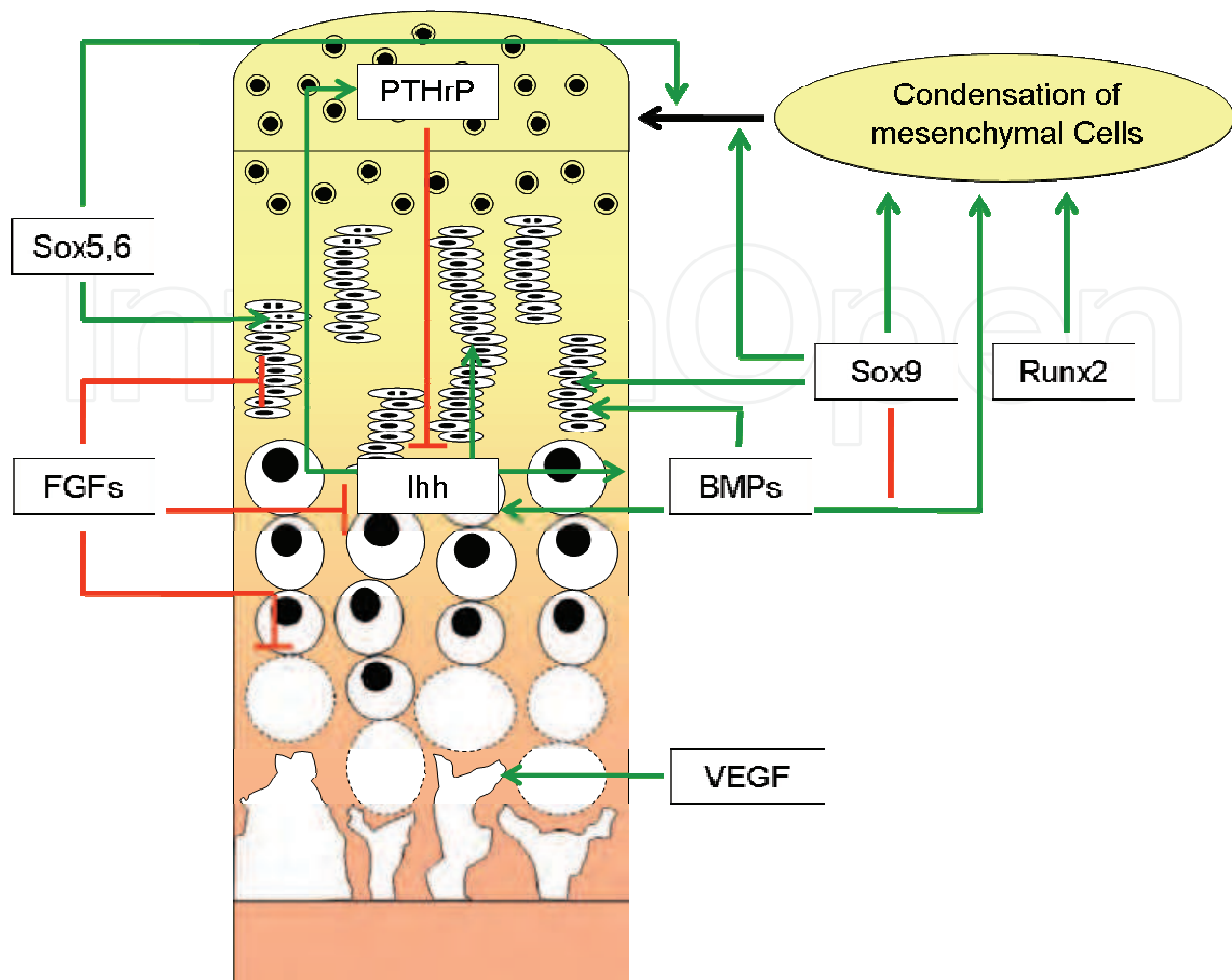


Fig. 3. Schematic depiction of the regulation of the growth plate with stimulating molecules (green arrows) and inhibitory molecules (red lines).

3.7 Prostaglandin E₂

Prostaglandin E₂ (PGE₂) is a metabolite of the arachidonic acid cascade, an evolutionary highly conserved family of lipid mediators. Prostaglandins are involved in numerous physiological and pathophysiological mechanisms, as well as in the regulation of developmental processes. In the latter context, PGE₂ also plays a significant role in skeletal development. In osteoblast-like cells, endogenous PGE₂ was shown to affect proliferation and differentiation by stimulation of DNA synthesis and alkaline phosphatase activity (Igarashi et al., 1994). Furthermore, PGE₂ influences the differentiation of chondrocytes as previously shown for the chondrocyte cell line RCJ3.1C5.18 (Lowe et al. 1996) and for rat growth plate chondrocytes (Schwartz et al. 1998, O'Keefe et al., 1992, Brochhausen et al., 2006). In addition to such findings in cell culture, the physiological role of prostaglandins was clarified by its stimulating effect on bone formation and increase in bone mass after systemic administration of PGE₂ to infants (Ueda et al., 1980) and animals (Suponitzki & Weinreb, 1998). Interestingly, also local administration of PGE₂ resulted in osteogenesis in situ (Yang et al., 1993, Marks & Miller, 1988). The cellular effects of PGE₂ are mediated by the binding to transmembranal G protein-coupled receptors. Four different EP receptors are known, which are linked to different intracellular signal transduction pathways (Narumiya

et al., 1999). The EP1 receptor is coupled to intracellular Ca^{2+} mobilization, while the EP2 and EP4 receptors increase intracellular cAMP accumulation. By contrast, EP3 inhibits intracellular cAMP accumulation. Regarding bone formation and bone resorption the EP4 receptor has been shown to be essential in terms of PGE_2 action in bone (Yoshida et al., 2002). In this respect the critical involvement of EP4 receptor in fracture healing was demonstrated very recently (Yoshida et al., 2002, Xie et al., 2009). Furthermore, EP2 and EP4 receptors were shown to be required for PGE_2 -dependent chondrocyte differentiation (Miyamoto et al., 2003).

Despite their obvious role in bone and cartilage metabolism, prostaglandins are not in the main focus regarding potential tissue engineering strategies of bone and cartilage.

Name	Substance class	Receptors	Effect	Reference
BMP	Protein growth factor	BMPRI-A, BMPRI-B	Cartilage & bone formation	Grimsrud et al. (2001), Pizette & Niswander (2000)
FGF	peptide growth factor	FGFR1, FGFR2, FGFR3	Mesenchymal condensation, Osteogenic differentiation	Kronenberg (2003), Hassan et al. (2007) Krejci et al. (2007)
PTHrP	Signalling peptide	G-protein G_s and G_q receptors	Chondrocyte proliferation	Lee et al. (1996)
Ihh	Signalling protein	Patched-1, Smo	Chondrocyte proliferation	Kronenberg (2003)
VEGF	Protein growth factor	VEGF-R	Vascularization, ingrowth of osteoblasts & chondroblasts	Gerber et al. (1999)
Sox	Transcription factor	DNA-binding	Chondrocyte differentiation & proliferation	Lefebvre et al. (1998) Akiyama et al. (2002)
Runx 2	Transcription factor	DNA-binding	Matrix mineralization, chondrocyte differentiation	Inada et al. (1999) Kim et al. (1999), Takeda et al. (2001), Ueta et al. (2001)
PGE_2	Lipid mediator	G-protein receptors, EP1, EP2, EP3, EP4	Chondrocyte proliferation, bone formation	Brochhausen et al. (2006) Xie et al. (2009)

Table 1. Summary of important growth factors and signalling molecules with relevant impact for the regulation of proliferation and differentiation of growth plate chondrocytes.

4. Experimental Setup

A principal aim of our research efforts was to develop an integrated release-system in an oriented scaffold for cartilage tissue engineering. Regarding the scaffold, one goal was to imitate the physiological fibre orientation of articular cartilage to achieve a topographically beneficial microenvironment. Articular cartilage represents a highly organized tissue not

only at the cellular level, but also at the level of extracellular matrix components. Based on the spatial distribution, the morphological characterization and the biochemical activity of cells three zones are distinguishable in the articular cartilage (fig. 4): the superficial zone with flat aligned chondrocytes and perpendicularly arranged fibres, the middle zone with clusters of round shaped chondrocytes in a scaffold of parallel aligned collagen fibres and the deep zone with calcified cartilage.



Fig. 4. Histomorphology of articular cartilage of the rat (HE, x200) with schematic depiction of the fibre arrangement.

Reviewing the previously described signalling molecules, there are several reasons for the use of PGE_2 in cartilage tissue engineering: First of all several *in vitro* and *in situ* data demonstrate an important role for PGE_2 in cartilage development. Furthermore, this molecule, its metabolism, biological effects and possible side effects are already well understood and investigated. Finally, this substance is already on the market since decades, and is thus available in pharmaceutical quality and for a price less expensive than many of the modern developments. Since PGE_2 has a short half-life, which ranges under physiological conditions between 30 seconds in the cardiovascular system and 35 minutes in amniotic fluid (Bygdeman 2003, Ghodgaonkar et al. 1979) we established a release system for this molecule as an important prerequisite for its potential use in tissue engineering strategies. As release system we developed poly-D,L-lactide-co-glycolide (PLGA) microspheres, which were originally designed for the expansion of chondrocytes (Gabler et

a., 2007). Polylactide and its polymers are well established as drug release systems and are already in clinical use. Some examples for polylactide based release systems which are already in clinical application or which are used in animal models are presented in Table 2, indicating that various substances can be integrated in corresponding systems. Thus, the polymer used in our experiments has also been shown to be suitable for the integration and controlled release of further potential signalling molecules or growth factors. For the analyses of the PGE₂ levels a novel sample preparation procedure was developed based on a previously published gas chromatography tandem mass spectrometry (GC-MS/MS) method for prostaglandin detection (Schweer et al., 1994). The modification of this method which is demonstrated in detail elsewhere (Watzer et al., 2009, Brochhausen et al., 2009) was necessary to determine the incorporated PGE₂ levels.

The combination of two different material approaches for scaffold and release systems was given by the idea to create a veritable toolbox which permits the use of different factors in the same three-dimensional scaffold material combined with a systematic analysis of their effects.

Active Agent	Release systeme	Indication	Reference
TGF-beta 1	PLGA (scaffold)	Cartilage/bone growth	Gombotz et al. (1994)
BMP	PLGA (scaffold)	Bone growth	Ferguson et al. (1987), Johnson et al. (1988)
VEGF	PLGA-PEG (microspheres)	Angiogenesis	King & Patrick (2000)
PGE ₁	Lipid-microspheres	Vasodilatation	Kanamaru et al. (1998)

Table 2. Summary of active agents integrated in a PLGA related release-system, which are either used in animal models or already in clinical application.

5. Research

5.1 Scaffold design

From the biomaterial point of view the basis for the scaffold system was the development of a processing route for a material with similar structural, biochemical and mechanical properties as native articular cartilage. The basic approach and methodology for the synthesis route is based on a directional freezing technique which can be further combined with an electrolytic process (Zehbe et al., 2005). For this purpose, a gelatin solution was exposed to an electric field which leads to an arrangement of the fibres along the gas bubble formation. The container with the gelatin solution was positioned on the cold side of a peletier element. Thus, variation of the applied temperature was possible. A detailed description of the combination process which adapts an electrolytic decomposition process of the gelatin solution to introduce another pore fraction prior to freezing is precisely described in Zehbe et al. (2004, 2005). Freezing led to a stabilization of the fibre arrangement along the direction of ice crystal formation. After complete freezing, the sample was freeze dried to remove the ice crystals. Since a non-cross linked gelatin-based sample would rapidly dissolve under the fluid conditions *in vitro* or *in vivo*, the material was finally cross-

linked to guarantee chemical stability of the material. As a result, a porous network was achieved after complete removal of the formed ice crystals.

Interestingly, the degree of directionality of the pores as well as the pore size is dependent on the freezing temperature, the ion content and the gelatin concentration. Thus, scaffolds with suitable directional pore channels could be synthesized at lower freezing temperatures without additional ions, while scaffolds with larger, non-directional pores could be made using higher freezing temperatures and elevated ion contents (fig 5a). In addition, based on this original scaffold processing route, we have introduced a hydroxyapatite layer to mimic the subchondral bone and address the potential application in tissue engineered-based treatment of deep cartilage defects (fig 5b).

Furthermore, we integrated in this scaffold drug release components in the form of PLGA-microspheres, which themselves were further enhanced by the integration of an active agent to meet so-called 3rd generation tissue engineering requirements.

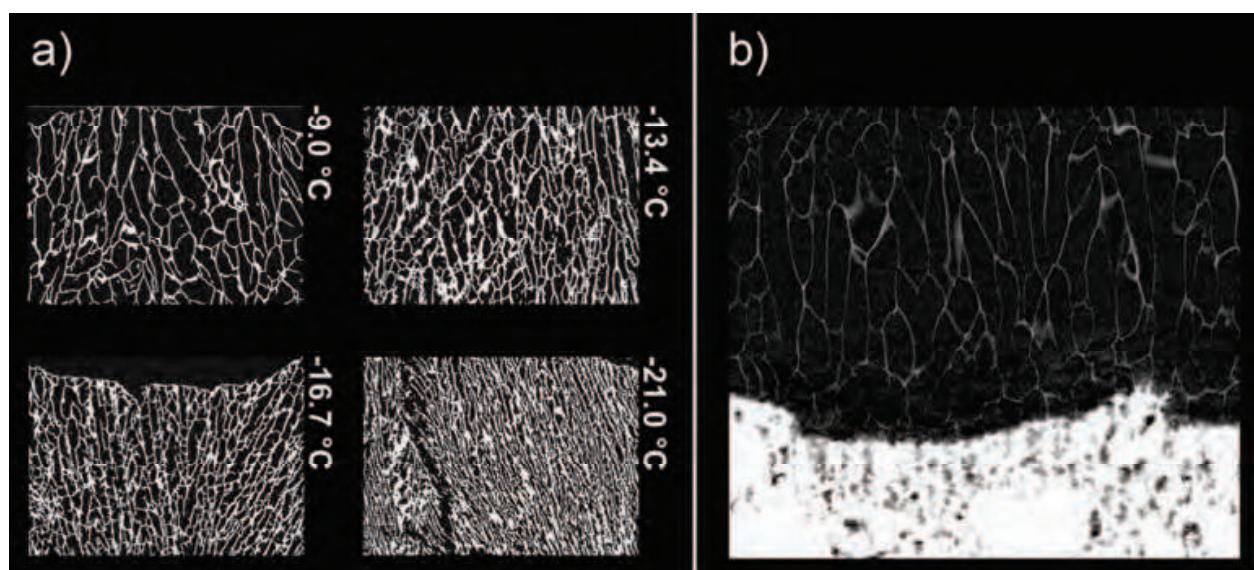


Fig. 5. X-ray tomographic slice representations of scaffolds with varying pore-channel networks (a) and sliced representation of a scaffold featuring hydroxyapatite as bone-like layer (b) (adapted from Zehbe et al., 2007).

5.2 PLGA- release system for prolonged PGE₂ release

As already mentioned above, the possible use of PGE₂ is highly limited due to its short half-life under physiological conditions. Therefore, for tissue engineering strategies this molecule should be used in the form of a release-system to guarantee a prolonged bioavailability. With respect to the microsphere scaffold modification we adapted an emulsion-based synthesis pathway which is described in detail by Brochhausen et al. (2007, 2009). In these protocols the microspheres were synthesized using a co-solvent emulsification method. The PLGA (Resomer RG859SR, Boehringer Ingelheim) used in our experiments had a lactide-glycolide-ratio of 85:15, a residual monomer content of < 2.0 % and an inherent viscosity of 2.5 - 3.5 dl/g. In the first step, 0.1 g PLGA granules were dissolved in 1.0 CHCl₃. Then, PGE₂ was dissolved in hexafluoroisopropanol (HFIP) in a concentration of 1.0 mg/ml. The PLGA solution was mixed with the PGE₂ solution giving the following composition: 0.1 g PLGA, 3.5 µg PGE₂, 1.0 ml CHCl₃ and 3.5 µl HFIP. To allow the complete removal of the organic

solvents by evaporation, the resulting solution was stirred over night in 0.5 % polyvinyl alcohol at 300 rpm with the help of a multi-position magnetic stirrer (RO 15 power Ikamag, IKA, Germany) according to the adaption of a previously described method (Gabler et al., 2007). Finally, the resulting microspheres were washed in sterile deionized water.

The characterization of the microspheres especially with respect to their size distribution was performed according to a protocol developed in our research group: In brief, SEM-images showing approximately 50 to 100 well dispersed microspheres were imaged top down and were then analyzed with the software ImageJ according to Gabler et al. (2007). In addition, the degradation changes regarding mass loss, pH-shift and the morphological changes of the microspheres in cell culture medium were documented over a timeframe of up to 100 days. Regarding the release profile we were able to show the kinetics of PGE₂ release over a period of 154 hours as measured by gas chromatography-mass spectroscopy (GC-MS/MS) and mass spectroscopy (Brochhausen et al., 2009). For this purpose the method of Schweer et al. (1994) was adapted. An important advantage of this method is that the structurally and thus functionally intact molecule could be detected even at very low concentrations. As a result of our analyses, we were able to determine reliably the amount of incorporated PGE₂ in the PLGA microspheres. Interestingly, the stability of PGE₂ was pH dependent: Strong acidic or basic environments reduced the half-life from 300 hours (pH 2.6 - 4.0) to below 50 hours at pH 2.0 or pH 8.8. Furthermore, we showed that the half-life of incorporated PGE₂ is highly temperature dependent (fig. 6). Thus, the half life could be increased from 70 days at 37 °C to 300 days at 8 °C. In our kinetic study we demonstrated a gradual concentration increase of PGE₂ in DMEM over the first 13 h, followed by a concentration plateau for 35 h and a slight decline of the PGE₂ concentration after the 48 h time point.

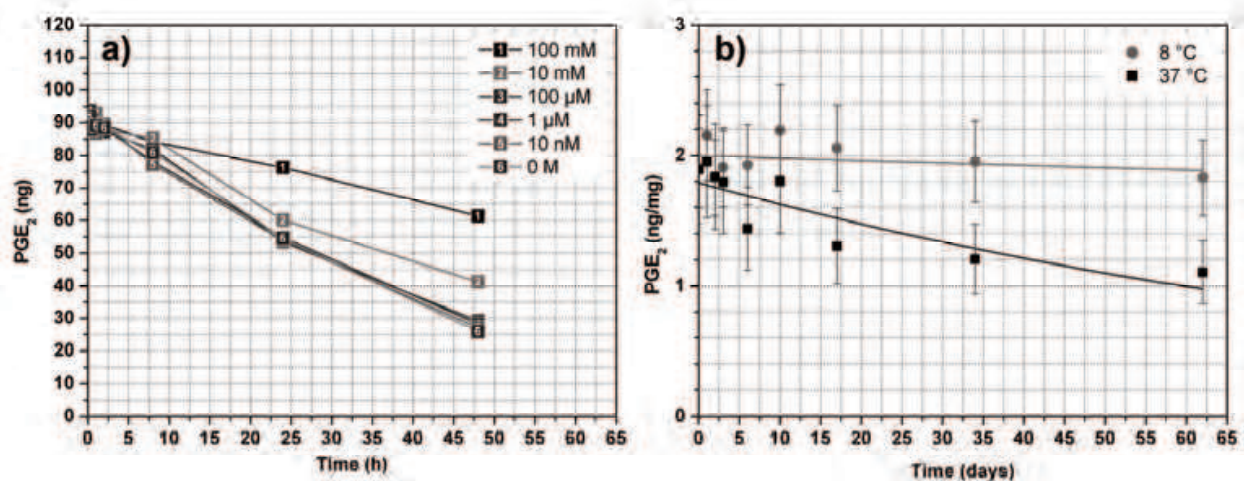


Fig. 6. pH dependent (a) and temperature dependent stability of PGE₂ (b), graphs adapted from Watzer et al., 2009.

For the used PLGA-type with a lactide-glycolide-ratio of 85:15 we hypothesized that the PGE₂ is primarily released from the outer shell of the microsphere as depicted in figure 7. Our image shows a microsphere after degradation for 80 days, as well as a superimposed graph of the kinetic experiments. After 80 days in cell culture medium the interior of the sphere is highly degraded, while the shell is almost intact. Further analysis with SR- μ CT

revealed that the microspheres consist of a porous shell and a compact core. These findings could be confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM) which clearly showed the internal and external structure of the microspheres with a distinct nanostructure of the polymeric phase and both nano- and micro-porosity (Watzer et al., 2009). Thus, the rapid increase of PGE₂ could be the result of the release out of the porous shell, whereas the later PGE₂ values could be the result of the release out of the core. Regarding the degradation of the microspheres we hypothesized that the slow erosion of the surfaces results in a semi-permeable membrane which delays the release of degradation products and thus results in autocatalytic processes, amplifying the degradation inside the material (Heidemann et al., 2002, Maspero et al., 2002, Gabler et al., 2007).

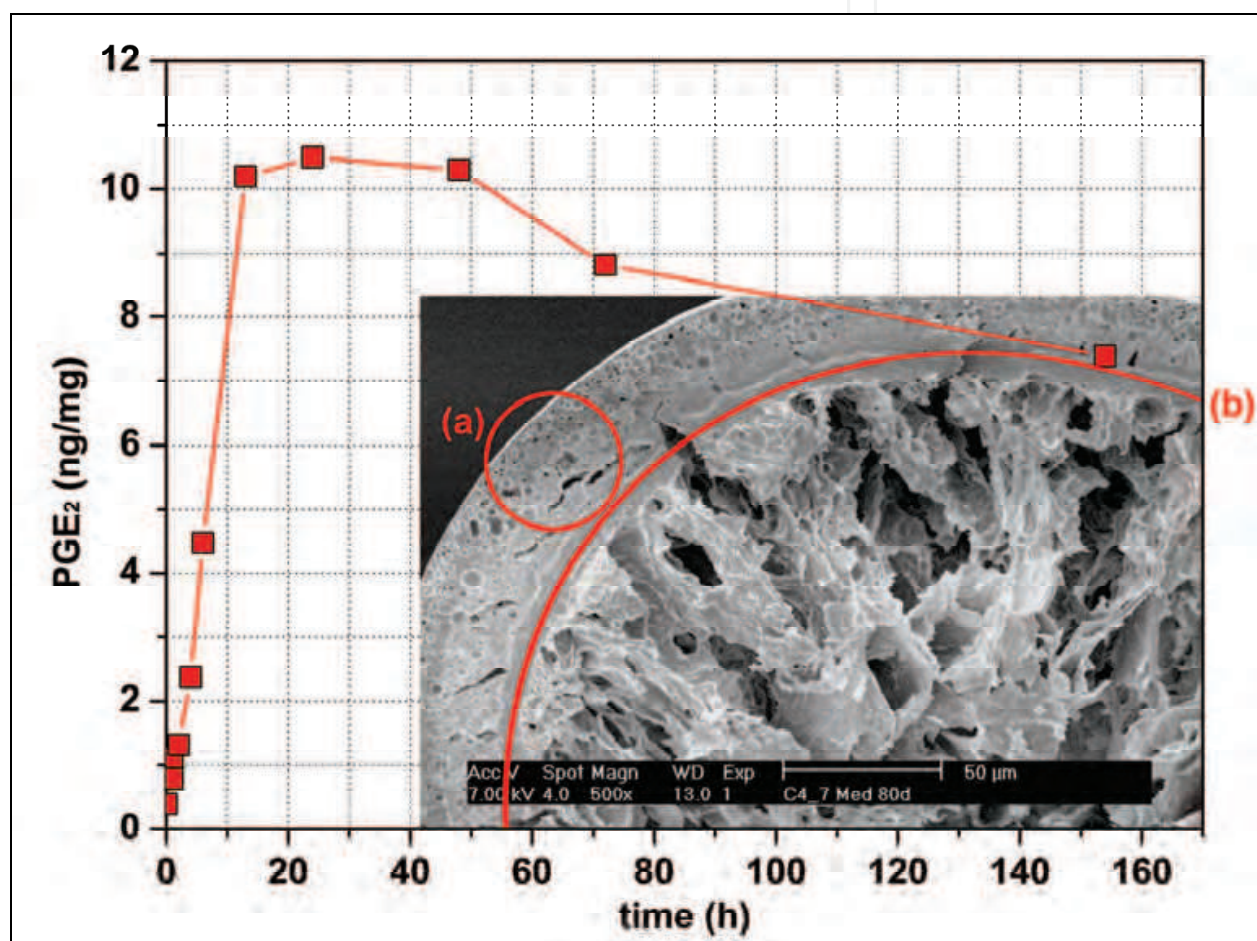


Fig. 7. PGE₂ release profile from PLGA microspheres and proposed release mechanism derived from SEM investigations. Initial release from the outer shell (a) and sustained release from the core after more than 160 h (b).

For the combined drug-release-scaffold system PGE₂-loaded microspheres were added to the gelatin solution and stirred until the gelatin gelled. By this procedure sedimentation of the spheres could be prevented so that a homogeneous distribution of microspheres could be reached without negatively affecting the casting of the gelatin in the freezing apparatus. After the freezing process, the microsphere-modified frozen gelatin samples were freeze dried and chemically cross linked as described above. We analysed the distribution of the

microspheres by high resolution X-Ray tomography as described in detail in the chapter by Zehbe et al. in this book.

5.2 Chondrocyte isolation and Cell Seeding of Scaffolds

For the cell culture experiments with the release-scaffold system bovine chondrocytes were used. For this purpose, fetlock joints of 3-4 months old calves were obtained directly after slaughter and processed under aseptic conditions. Articular cartilage chondrocytes were harvested aseptically from dissected flakes of the full thickness cartilage. After harvesting the cartilage flakes were washed twice for 15 min with Tyrode's balanced salt solution (TBSS) containing penicillin, streptomycin and amphotericin B. The cartilage flakes were then predigested with 1 mg/mL of pronase (Roche Diagnostics GmbH, Mannheim, G) on a magnetic stirrer at 37°C for 2 h, followed by washing with TBSS. Afterwards the specimens were digested with 600 units/mL of collagenase type II (Worthington Biochemical Corp, Lakewood, NJ) in DMEM by stirring at 37°C for a maximum of 15 h. The suspension was then filtered, and the isolated chondrocytes were washed 3 times with DMEM containing 10% fetal bovine serum (FBS). The viability of the isolated cells was determined using the trypan blue exclusion method. The cells were cultured in 175 cm² culture flasks at 5% CO₂, 95% humidity and 37°C in DMEM containing 10% FBS. Chondrocytes were cultured in monolayers until confluence and then seeded onto the scaffolds. Cell-seeded scaffolds were cultured in 12-well tissue culture plates in DMEM supplemented with 10% FCS, nonessential amino acids, and 40 µg/mL of l-proline. After 2 days, 50 µg/mL of ascorbic acid was added. The medium was changed three times per week. Cell scaffold constructs were analyzed after 8 days of culture.

In these experiments the cultured cells showed a round shaped chondrocytic phenotype as seen by histological examination. Regarding the distribution of cells the majority of cells was detectable in the upper half of the scaffold. In this area cell clusters consisting of two or more cells could be seen, which are typical for the middle zone of articular cartilage. With the help of alcian blue staining, the production of extracellular matrix components was demonstrated especially surrounding the cell clusters (fig 8).

In further experiments with cultured human chondrocytes from patients who underwent implantation of endoprosthesis due to osteoarthritis, preliminary data revealed that in the presence of low dose PGE₂ the seeded cells showed a chondrocytic, round shaped phenotype with arrangement of multiple clusters, whereas cells without PGE₂ showed a spindle shaped fibrocytic phenotype (Brochhausen et al., 2008).

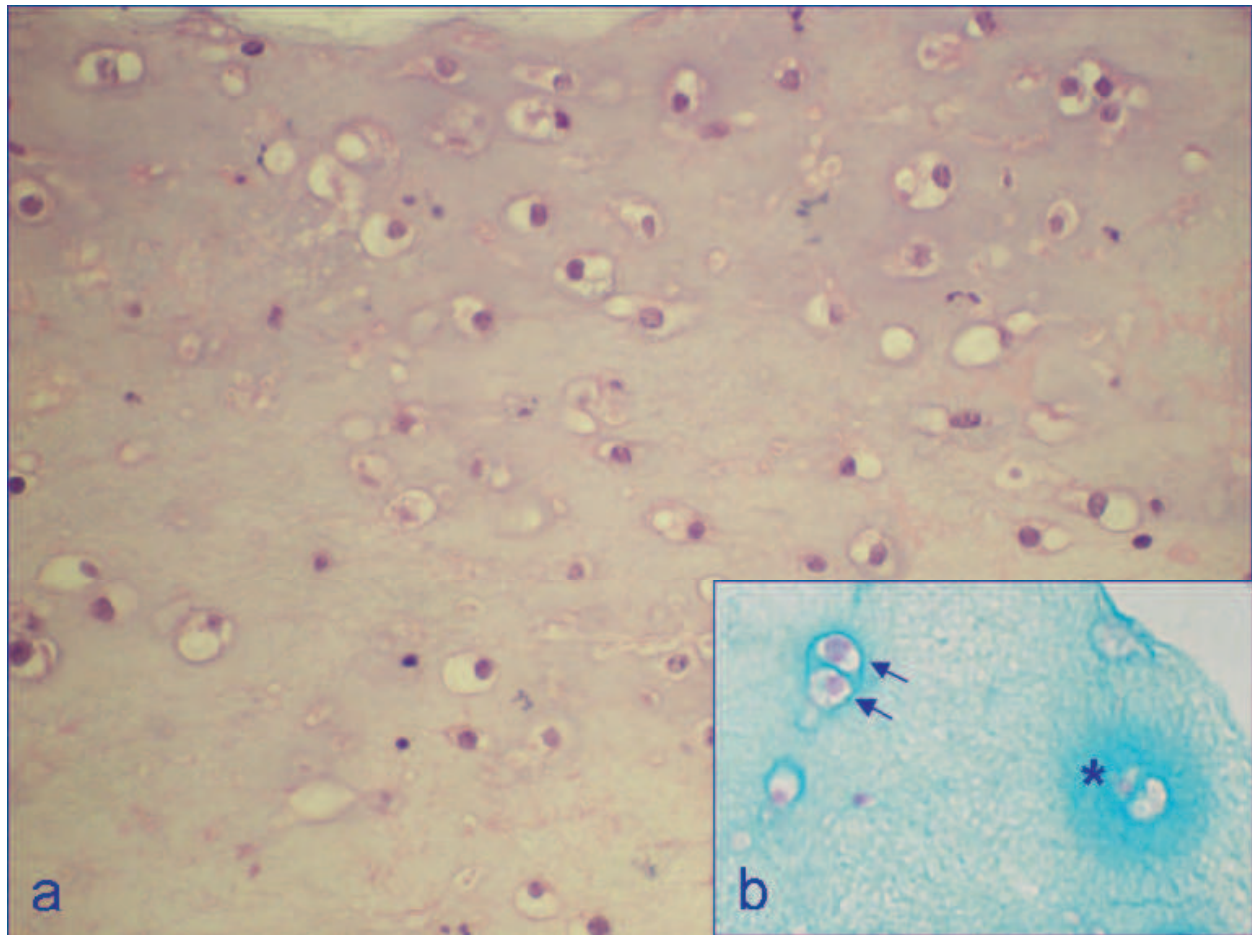


Fig. 8. Bovine chondrocytes cultivated for eight days in the combined release-scaffold system with round shaped chondroid phenotype (a, HE, x400), formation of chondrocyte clusters (b, arrow) and relevant extracellularmatrix production (b, alcian blue, x400)

6. Conclusion

In the present report we demonstrate that growth plate cartilage is a suitable model for strategies to target potential signalling molecules and growth factors for tissue engineering. Furthermore, we described the synthesis of a scaffold material with a structural design that gives ingrowing cells a pre-defined microenvironment similar to the cellular organization in native articular cartilage. Based on knowledge gained from the development of bone we used PGE_2 as a signalling molecule for cartilage tissue engineering strategies. In this context we firstly immobilized this molecule in polyester-based (PLGA) microspheres and then incorporated these in our gelatin-based orientated scaffold. The present experiments confirmed the prolonged release of PGE_2 from the PLGA microspheres and the beneficial effects of low dose PGE_2 for the phenotype of bovine and human articular chondrocytes seeded on the three-dimensional scaffold.

Since PLGA release systems are already used for various substances the present microsphere-scaffold system opens the perspective for the incorporation of further signalling molecules and growth factors. Furthermore, our construct facilitates the systematic analysis of different factors influencing the proliferation and differentiation of chondrocytes in an identical three-dimensional environment. For further tissue engineering

applications it is of interest to create gradients of signalling molecules and growth factors within the scaffold by establishing a graded microsphere distribution.

In conclusion, we demonstrate in this contribution an innovative scaffold material with properties of a biphasic composition and the incorporation of signalling molecules with the perspective to improve our understanding of the triad of cells, materials and signalling molecules as essential elements for tissue engineering strategies.

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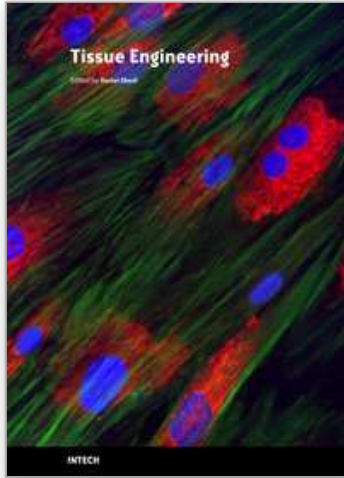
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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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