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PREDICTIVE VALUE OF *IN VITRO* AND *IN VIVO* ASSAYS IN BONE AND CARTILAGE REPAIR — WHAT DO THEY REALLY TELL US ABOUT THE CLINICAL PERFORMANCE?

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22.1. INTRODUCTION

The continuous increase of life expectancy leads to an expanding demand for repair and replacement of damaged and degraded organs and tissues. Recent completion of a first version of the human genome sequence is a great breakthrough for the field of pharmaceutics. It is conceivable that new developments in pharmaceutical research will result in a large number of novel and improved medicines. A similar development is expected in the field of biomaterials designed for bone and cartilage repair and replacement. Spinal fusions and repairs of bone defects caused by trauma, tumors, infections, biochemical disorders and abnormal skeletal development, are some examples of the frequently performed surgeries in the clinic. For most of these surgeries, there is a great need for bone graft substitutes. Similarly, the number of patients worldwide experiencing joint pain and loss of mobility through trauma or degenerative cartilage conditions is considerable, and yet, few approaches employed clinically are capable of restoring long-term function to damaged articular cartilage[1, 2]. Therefore, new materials and techniques need to be developed.

This expanding number of newly developed biomaterials and techniques are accompanied by an increased need for high-throughput screening systems which are reliable in predicting the performance of the material or construct in the function it was developed for. An example of a recently developed high-throughput system is microscale screening of polymer-cell interaction by using microarrays [3].

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In this review, we attempt to provide answers to two questions regarding the reliability of the existing assays used in bone and cartilage repair strategies involving biomaterials: (i) what do *in vitro* assays really tell us about the *in vivo* performance? and (ii) what do *in vivo* assays tell us about the clinical conditions?

This review consists of two parts, one regarding bone- and one regarding articular cartilage repair and regeneration.

In the first part, we focus on limitations of the existing, frequently used assays to test the performance of synthetic biomaterials for bone repair and regeneration. We provide an introduction in *in vitro* bone formation assays in general, and an overview of organs and cells which are commonly applied in the *in vitro* bone formation assays. We then give an overview of a number of *in vitro* and *in vivo* studies performed with similar materials for bone repair, in order to investigate correlation between their results. Subsequently, we address the shortcomings of the existing *in vitro* assays and give some recommendation for their improvement. Finally, we give a short overview of different *in vivo* assays used to test biological performances of biomaterials for bone repair and regeneration.

The focus of the second part of this paper is shifted from biomaterials alone to tissue-engineered hybrids for articular cartilage restoration, as in this, rather new field, the use of biomaterials alone is rare. We first give a short review of biomaterials and cells which are frequently used for development of tissue engineering hybrids for cartilage repair. We then discuss different parameters which can be of influence when combining cells and biomaterials to produce tissue engineered hybrids. We point out some drawbacks of the existing *in vitro* assays which are used to predict the *in vivo* performance of the hybrid constructs and elaborate on possible ways to improve the existing assays. Finally, we review different *in vivo* animal models, together with their advantages and shortcomings in order to shed light upon their predictive value for the clinical setting.

22.2. BACKGROUND ON *IN VITRO* BONE FORMATION ASSAYS

In research into new bone graft substitutes, two types of preclinical assays are used in general: *in vitro* assays using a cell- or an organ culture system (i.e. *in vitro* bone formation assays) and *in vivo* assays, using experimental animal models. *In vitro* assays have initially been developed to study the influence of growth factors and hormones on attachment, proliferation, differentiation and mineralization of cells for example. Subsequently, investigators started to use these *in vitro* assays in biomaterials research. Instead of studying the influence of e.g. growth factors on the differentiation of cells, the behavior of cells in the presence of the testing material is studied. However, in general, it is ignored that the *in vitro* setting may significantly be changed by the presence of a material due to e.g. material-cell culture medium interaction. If such an interaction is not expected *in vivo*, it raises the question of what the predictive value of the *in vitro* assay is for the *in vivo* performance of the material.

Besides the increasing need for reliable *in vitro* assays to test biomaterials prior to implanting them in animals and humans, investigators need tools which are helpful in unraveling mechanisms of complex *in vivo* phenomena regarding bone formation. *In vitro* assays are attractive because of their simplicity, but, at the same time, their simplicity is an important limitation. It is of course impossible to fully simulate the *in vivo* situation in a culture dish and yet in many publications, rather strong conclusions about the *in vivo*

performance of biomaterials and about mechanisms of complex biological phenomena are drawn from the *in vitro* studies.

22.2.1. Cells and Cell Sources

In vitro bone formation assays have initially been developed as tools to study the effects of hormones and cytokines in a controlled environment [4]. Despite the inherent diversity in these systems, most of them share some common features. For example, the basic culture environment (e.g. medium composition, serum type and concentration, supplements, temperature and antibiotics) and methods of routine maintenance (e.g. feeding, subculturing, cloning) are very similar in all systems. Gronowicz and Raisz [4] have given an overview of the culture conditions which are generally applicable for different *in vitro* bone formation culture systems. The fact that they give a simplified reflection of the *in vivo* situation and allow for the research in a controlled environment are primary reasons for the use of *in vitro* assays. In addition, from financial and ethical point of view, *in vitro* assays are preferred above the *in vivo* ones.

The existing bone formation assays can be divided into two groups: organ culture systems and bone cell culture systems.

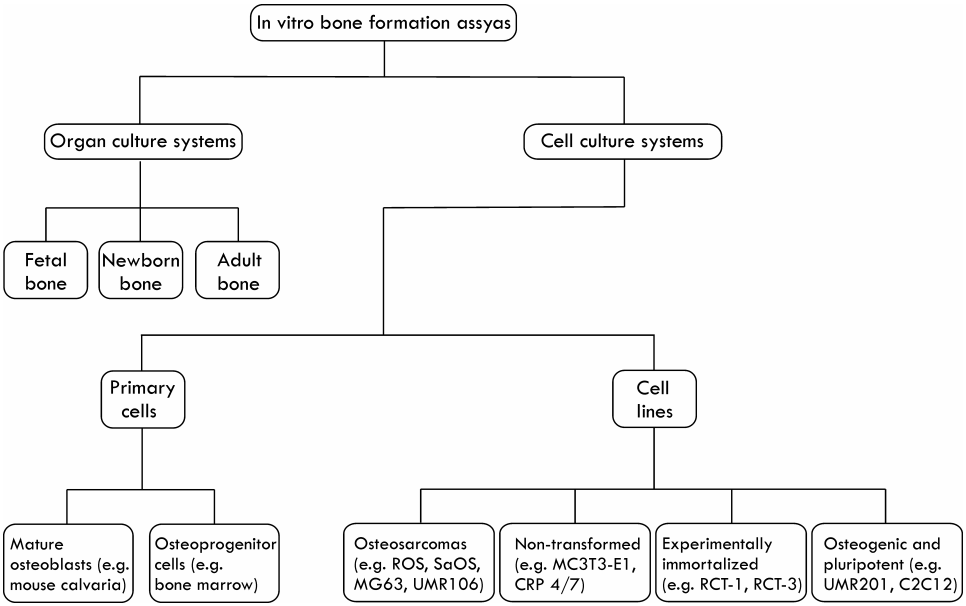

Figure 22.1. Overview of *in vitro* bone formation assays.

As reviewed by Gronowicz and Raisz [4], tissues used for bone formation assays in bone organ culture systems vary in source and age including fetal, newborn and occasionally adult bone. Chicken, mouse and rat bones are most common, although human bone fragments have also been used. Bone from calvaria and limb are the most frequently cultured tissues. Fetal calvaria are characterized by intramembraneous bone

formation, while growth of long bones is mainly endochondral. However, as intra–membraneous and endochondral bone may have different responses to hormones, growth factors and environmental conditions, most *in vitro* organ culture systems have limitations and may not give a similar response as endochondral bone and adult bone cells.

In addition to organ culture systems, the *in vitro* isolation and culture of bone-derived cell populations has substantially enhanced our ability to understand factors important for the proliferation and differentiation of cells of the osteogenic lineage. As recently reviewed by Kartsogiannis and Ng [5], commonly employed model systems include either primary cultures of osteoblastic cells derived from fetal calvaria and subperiosteal fetal long bones, or established cell lines that can be divided into clonal cell lines from cells isolated from bone tumors (osteosarcomas), non-transformed cell lines, experimentally immortalized cell lines and bone marrow cultures. Figure 22.1. gives an overview of *in vitro* bone formation assays.

22.3. *IN VITRO* MODELS FOR ASSAYING BONE GRAFT SUBSTITUTES

As mentioned previously, the expanding development of (synthetic) biomaterials for support, replacement and regeneration of bone has created the need for *in vitro* systems in which the potential *in vivo* performance of these materials can be assayed. *In vitro* cell- and organ culture assays are in the first place used to investigate the "safety" of the material in terms of cytotoxicity and biocompatibility for example. In addition, *in vitro* bone formation assays are used in order to predict the performance of the material *in vivo* in its role of e.g. bone filler. In this case, the potential osteoconductivity of the material is tested. Finally, *in vitro* cell culture systems are used to study complex and not yet fully unraveled "biologically driven" phenomena such as osteoinduction. Below, we give a few examples of *in vitro* studies in which materials' cytotoxicity, osteoconductivity and osteoinductivity were assayed. In addition, the results and authors' conclusions drawn from these studies are compared to the results *in vivo*, where similar materials were tested.

22.3.1. Cytotoxicity

Hyakuna et al. investigated changes in calcium-, phosphate-, magnesium- and albumin content of cell culture medium after immersion of different biomaterials [6]. The results of this study showed that monocrystalline and polycrystalline alumina ceramics did not have any influence on the surrounding medium. Two types of apatite containing glass ceramics (apatite and wollastonite-containing glass ceramic (AW-GC) and apatite-, wollastonite- and whitlockite-containing glass ceramic (AW-CP-GC)) showed a slight decrease of phosphorus and a slight increase of calcium ion concentration in the culture medium. Hydroxyapatite (HA) ceramics sintered at 600°C and 900°C with a very high specific surface area showed a high and rapid adsorption of calcium- and phosphate ions and albumin from the medium. Changes of calcium and phosphate concentrations of the medium were suggested to be the reason for the poor attachment of V79 Chinese hamster fibroblasts, and hence for a higher apparent cytotoxicity of the HA ceramics sintered at

600 and 900°C and the two glass ceramics as compared to the tissue culture plastic and alumina ceramics.

Suzuki et al. prepared ceramics with calcium to phosphorus (Ca/P) ratios varying from 1.50 to 1.67 by mixing different amounts of HA and tricalcium phosphate (TCP) ceramics and observed variations of zeta-potentials of different surfaces after immersion in the cell culture medium [7]. Decrease of calcium- and phosphate ions in the culture medium was always observed, but its intensity depended on the Ca/P ratio of the ceramics and so did the change of the pH of the medium. Changes of the ions concentrations and pH of the medium were suggested to be of influence on the attachment of L-929 cells on the ceramic surfaces, and thus on the cytotoxicity of the material.

Knabe and coworkers performed a similar study, in which they compared attachment and proliferation of rat bone marrow cells (RBMCs) on highly resorbable calcium-phosphate (CaP) ceramics [8] and on glassy materials with different rates of resorbability [9]. Interestingly, while in the studies described previously authors observed a decrease of calcium- and phosphate ions from the medium and suggested this decrease to be the reason for poor attachment and growth of the cells, Knabe and coauthors suggested that the inhibitory effect on cellular growth on some of their materials was associated with an increased concentration of phosphorus ions released into the medium by these materials and the formation of a phosphorus-rich layer on their surface. Daily refreshment of the medium increased the osteoblast attachment on some, but not on all tested ceramics.

In later work of this group, in which highly resorbable CaP cements and CaP ceramics were compared, it was suggested that increased levels of phosphate- and potassium ions, decreased levels of calcium ions and hence elevated pH of the medium were reasons for poor attachment and proliferation of RBMCs.

The above described examples of studies in which safety of materials in terms of cytotoxicity was tested all emphasized the presence of the biomaterial-cell culture medium interaction, which seems to be responsible for, or at least of influence on the behavior of cells. Although material-medium interactions are sometimes of great influence on the behavior of cells *in vitro*, in the *in vivo* environment they might be less important if observed at all, as, unlike in a culture dish, in the body there is a continuous supply and thus refreshment of nutrients and body fluids. For the cytotoxicity tests, this probably means that the *in vitro* settings give a more "negative" reflection of *in vivo* situation.

22.3.2. Osteoconduction

Osteoconduction, defined as "spreading of bone over the surface proceeded by ordered migration of differentiating osteogenic cells" [10], is supposed to be driven by physico-chemical properties of the material, having its origin in dissolution/reprecipitation or precipitation of a CaP layer on the surface of the material [11, 12]. An important aspect is thereby the direct bonding of bone to the material without fibrous tissue deposition, so-called contact or bonding osteogenesis [10]. In a few studies, these properties of the materials were tested first *in vitro* and then *in vivo*.

De Bruijn et al. used an *in vitro* RBMCs culture system to study various types of CaPs [13]. Besides the elaboration of different interfaces, mineralization occurred at a later

time on slow degrading materials such as fluorapatite (FA), than on fast degrading materials such as TCP. Authors therefore suggested that a more dynamic interface is formed on degrading materials that could be favorable for bone formation to occur. This hypothesis was further tested by implanting various plasma-sprayed CaP coatings in rat femoral bone for relatively short period of time[14]. The results of this study indeed suggested that initially a greater amount of bone was formed on fast degrading amorphous HA as opposed to the slow degrading highly crystalline HA.

A recent report by Wang and coworkers described a comparison of proliferation and differentiation of SaOS-2 osteoblastic cell line on HA ceramics sintered at three different temperatures (1200°C, 1000°C and 800°C) [15]. Results of this study showed that cell proliferation rate on HA ceramic sintered at 1200°C was the greatest. In addition, Bone Sialoprotein (BSP), Osteocalcin (OC) and Osteonectin (ON) protein levels after 12-day-culture were significantly higher on HA sintered at 1200°C as compared to HA ceramics sintered at 1000°C and 800°C respectively. Authors therefore concluded that HA ceramic sintered at 1200°C, which had a significantly lower specific surface area than the other two ceramics, was the best candidate to be used as a bone graft. They suggested that the ceramic sintered at higher temperature possibly had a less reactive surface and hence a lower cytotoxicity as compared to the other two tested ceramics. These results were in accordance with the results of the *in vitro* study of Hyakuna et al. [6]. In a study by our group [16] however, biphasic calcium phosphate (BCP, consisting of HA and β-TCP) ceramics sintered at 1150°C and at 1300°C were implanted in a critical-sized iliac wing defect of goats. Significantly more bone was found in the orthotopically implanted BCP sintered at 1150°C as compared to BCP sintered at 1300°C [16]. The two materials had similar compositions and macroporosities and they only differed in their microporosities. BCP1150 with its higher microporosity and hence higher specific surface area in comparison with BCP1300 was suggested to have a higher surface reactivity, which was consequently the reason for a higher bone regenerative potential. These *in vivo* results were thus in conflict with the *in vitro* data given by Hyakuna et al. [6] and Wang et al. [15], in which ceramics sintered at higher temperatures showed a more pronounced cell proliferation and osteogenic differentiation.

The above described are only a few examples of studies in which *in vitro* bone formation assays were used to predict the performance of biomaterials *in vivo*. As can be seen, in some studies, *in vitro* results completely fit the *in vivo* results, while in the others, differences observed *in vitro* could not be found *in vivo* or were in full contrast with the *in vivo* data. In the reviewed studies, both *in vitro* and *in vivo* studies were performed with similar biomaterials. This is, however, not always the case. Sometimes, only *in vitro* results are presented, and authors use these to draw conclusions on the performance of materials *in vivo*, which makes it impossible to elaborate on the predictive value of the *in vitro* assays.

22.3.3. Osteoinduction

Osteoinduction is an even less understood phenomenon as compared to osteoconduction. In the sixties, osteoinduction was defined as “the differentiation of the undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells” [17]. In other words, osteoinductivity is

the ability of a cytokine or a material to induce bone formation ectopically. Extensive research of Urist and others led to the conclusion that a discrete protein within the demineralized bone matrix (DBM) was the sole inducer of bone formation. This finding was published in 1971 and this protein was named Bone Morphogenetic Protein (BMP)[18]. BMP was shown to be involved in the bone formation cascade of chemotaxis, mitosis, differentiation, callus formation and finally bone formation. Besides the BMP-driven osteoinduction, many investigators have shown that also some biomaterials that neither contain nor produce BMPs are also able to induce ectopic bone formation [19-25]. Despite the extensive research, the underlying mechanism of osteoinduction is still largely unknown, and reliable assays to study this mechanism are needed. Below, a few *in vitro* studies on osteoinduction are described.

Adkisson et al. [26] developed a "rapid quantitative bioassay of osteoinduction" by using SaOS-2 osteosarcomas and studied cell proliferation rates under the influence of DBM. The observed correlation between cell proliferation and osteoinduction was not strong. Osteogenic factors, like BMP are not commonly associated with mitogenic response.

Zhang et al. [27] and Wolfinbarger and Zhang [28] used human periosteal cells and human dermal fibroblasts to relate cellular ALP activity to DBM osteoinductivity. In these studies, the authors failed to show a clear correlation between *in vitro* assays and *in vivo* bone formation.

Carnes et al. used an immature osteoprogenitor cell line, 2T9 to investigate the effect of DBM on their differentiation [29]. They failed to show any effect on differentiation and concluded that there are no soluble factors being released from DBM into the culture medium.

Han et al. assayed Alkaline Phosphatase (ALP) activity of the C2C12 cells in a culture in presence of DBM, and succeeded correlating it with the *in vivo* bone formation [30]. The last study mimics the *in vivo* situation more than other described studies, although the expression of ALP is not the most sensitive marker for the osteogenic differentiation.

Regarding the mechanism of osteoinduction by biomaterials, a very limited number of studies are performed and published. In our group, an extensive number of studies have been performed *in vivo*. In addition, we have tried to perform a number of *in vitro* studies as well, however, their results were either inconclusive, or in contrast with the *in vivo* observations. A few examples of the performed studies are described below.

In an earlier published study [21], HA ceramics sintered at 1150°C and 1250°C together with biphasic calcium phosphate (BCP, consisting of HA and β-TCP) ceramics sintered at 1100°C, 1150°C and 1200°C were implanted intramuscularly in goats and we found that HA sintered at 1150°C induced bone formation intramuscularly, while no bone was induced by HA sintered at 1250°C. Furthermore, the amount of induced bone by BCP ceramics increased with decreasing sintering temperatures.

In another study [16], BCP ceramics sintered at 1150°C and at 1300°C were implanted intramuscularly in goats. Ectopic bone formation was only found in the BCP sintered at lower temperature [16]. The presence of microporosity in BCP1150 was suggested to be responsible for a higher osteoinductive potential in comparison with BCP1300 ceramic. In order to compare BCP1150 and BCP1300 *in vitro*, we cultured MC3T3-E1 osteblastic-like cells and C2C12 pluripotent mesenchymal cells (in presence and in absence of BMP-2) on BCP1150 and BCP1300 ceramics and consequently investigated the expression of various osteogenic markers using RT-PCR (e.g mRNA for ALP, Parathyroid Hormone

receptor (PTH-r), OC, ON, Osteoblast-Specific Factor 2 (OSF-2) and Osterix (Osx)). For both cell types, a higher expression of most markers was observed on BCP1300 than on BCP1150 (Figure 22.2.). Furthermore, the expression of these markers was the highest in cells cultured on tissue-culture (TC) plastic.

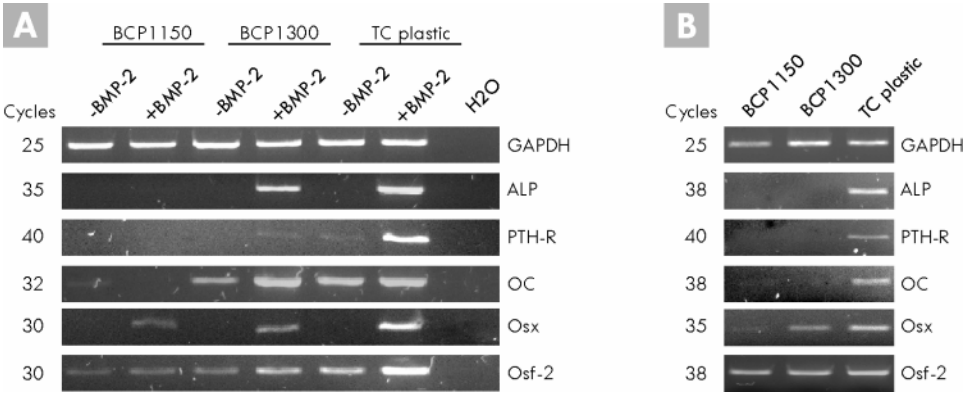

Figure 22.2. RT-PCR data showing the temporal expression of osteogenic mRNA by C2C12 cells cultured with and without BMP-2 (100ng/ml) (A) and MC3T3-E1 cells cultured without ascorbic acid (B) for 6 days on BCP1300 and BCP1150 discs (diameter 25mm, height 5 mm). The expression of most osteogenic markers by C2C12 cells (A) is increased when cells are cultured in presence of BMP-2. In both presence and absence of BMP-2, the expression of most markers is highest when the C2C12 cells are cultured on tissue culture plastic. Cells show a higher expression of osteogenic markers when cultured on BCP1300 as compared to BCP1150. Similar to C2C12 cells, the expression of all investigated osteogenic markers by MC3T3-E1 cells (B) is the highest on TC plastic, followed by BCP1300 and then BCP1150.

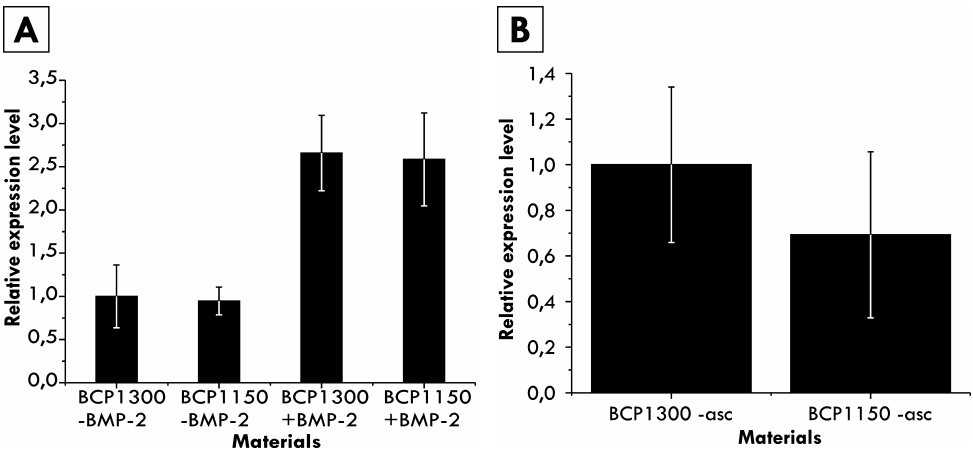

Figure 22.3. Q-PCR data (n=3) showing relative expression of Osteocalcin mRNA by C2C12 cells cultured with and without BMP-2 (100ng/ml) (A) and MC3T3-E1 cells cultured without ascorbic acid (B) for 6 days on BCP1300 and BCP1150 particles (1-2 μm). The Osteocalcin expression by C2C12 cells (A) is significantly increased when cells are cultured in presence of BMP-2 on both BCP1300 and BCP1150 ceramic. There are no significant differences in the Osteocalcin expression between cells cultured on BCP1300 and BCP1150 for neither cell type. However, the trend for both cell types is the same, namely a slight downregulation of Osteocalcin expression on BCP1150 as compared to BCP1300.

Measurements of calcium and phosphate contents of the medium after 3 hours of soaking showed a decrease of calcium concentration with 22% and a decrease of phosphate concentration with 18% in presence of BCP1300, while the decrease of calcium and phosphate concentrations in presence of BCP1150 were 62% and 60%, respectively. After 3 days of soaking, which is the normal time point at which culture medium is refreshed, calcium- and phosphate contents of the culture medium further decreased with 8% for both ceramics. No changes in calcium- and phosphate contents of the medium in the absence of ceramics were observed.

In order to decrease the change of the contents of the medium, we repeated the experiment with MC3T3-E1 and C2C12 cell lines by using considerably smaller (±100 times lower volume) amount of BCP1150 and BCP1300 scaffolds in the same volume of medium. This time, the amount of calcium decreased with 7% and the amount of phosphate with 11% in presence of BCP1300 scaffold after 3 days of soaking. Decrease of calcium concentration was 36% and that of phosphate 40% in presence of BCP1150. Although differences in the expression of OC (Figure 22.3., Q-PCR data) for MC3T3-E1 and C2C12 cells between BCP1150 and BCP1300 were smaller this time, the trend of expression remained the same. Differentiation of cells towards the osteogenic lineage was higher on BCP1300 as compared to BCP1150, while *in vivo* significantly more bone was induced by BCP1150 in comparison to BCP1300.

A similar study was performed with mouse embryonic stem cells (to be published separately), and interestingly, in this study, the expression of mRNA for OC and BSP was higher on BCP1150 as compared to BCP1300. Whether these results mean that the effect of the material is only visible in very early stages of differentiation, or simply that ESCs react differently to the changes of the medium caused by the presence of ceramics as compared to C2C12 and MC3T3-E1 cells, needs to be further investigated.

22.3.4. Limitations of *In Vitro* Models for Assaying Bone Graft Substitutes and Recommendations for Their Improvement

All examples described above suggest that the use of the existing *in vitro* assays in biomaterials research might not always be valuable. Sometimes, the *in vitro* data are completely in accordance with the *in vivo* findings, especially when rather simple physico-chemically guided processes are studied. In other studies, in which more complex, biologically driven processes are studied, *in vitro* and *in vivo* results are in full contrast with each other. The question that needs to be answered is what the cause of these inconclusive results is.

First of all it is important to note that in most cell culture and organ culture systems involving biomaterials there is, in addition to cell-biomaterial interaction, often a very important biomaterial-cell culture medium interaction which often markedly influences the outcomes of the study. In the *in vivo* environment these interactions might be less important if observed at all, as, unlike in a culture dish, in the body there is a continuous supply and thus refreshment of nutrients and body fluids.

Although most examples given above are studies performed on CaP containing biomaterials, the changes in the medium can also be caused by non-CaP materials (e.g. certain polymeric sponges [31], alumina ceramics [32], and porous titanium [33] scaffolds are

capable of forming a CaP layer when immersed in a CaP-rich environment). Release of calcium, phosphate, magnesium, and other ions from highly resorbable materials, uptake of different ions from the culture medium by a high surface area of a material, changes of pH and Z-potentials on the surfaces, formation of phosphorus and/or calcium rich layers on the surfaces, adsorption of all, or selected proteins from the serum-containing cell culture media, are only a few observations from these types of studies. Obviously, all these changes of the medium differ significantly between the tested materials and raise therefore the question if such *in vitro* systems are applicable for the comparative types of experiments. Different studies focus on comparing material A with material B by studying cell attachment, proliferation, differentiation and mineralization on their surfaces. However, if the interaction between material A and the culture medium is different from the interaction between material B and the culture medium, the cells will attach, grow and differentiate in different environments and can therefore not be compared with each other when similar biomaterial-body fluid interactions are not expected *in vivo*. In addition, changes which take place in the medium due to the presence of a biomaterial will influence different cell types in a different manner, which makes comparisons between different studies difficult, if not impossible.

In addition to taking into account possible side effects of the presence of biomaterials in *in vitro* cell culture systems, the choice of cells is of great importance for the reliability of the results. For example, if one would like to compare two biomaterials and be able to draw some conclusions regarding their potential performance as bone graft substitute, would the attachment and proliferation of primary rat osteoblasts then be the right assay knowing that *in vivo* osteoblasts are not the cells which are initially in contact with biomaterial surface? The choice is probably even more difficult when one is trying to investigate a largely unknown phenomenon *in vitro*, such as osteoinduction by BMPs or even less understood osteoinduction by biomaterials. Obviously, in order to study the mechanism of osteoinduction by biomaterials, it is probably not sufficient to choose osteoblasts or osteoblast-like cells as osteoinduction is the process of differentiation of cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells. Therefore, murine pluripotent mesenchymal C2C12 cells could be better candidates than osteoblasts. However, it is well-known that ectopic bone formation by biomaterials is only very rarely found in mice [34-37], making cells of murine origin possibly an inadequate choice. In addition, it is hard to decide whether the culture of C2C12 cells on osteoinductive biomaterials should be performed in presence or in absence of e.g. BMP-2, as it is suggested, but not yet proven [22, 38], that BMPs play a role in the process of osteoinduction by biomaterials. Similar questions of the choice of cell origin and culture conditions should be answered if one would choose to use ESCs to study the phenomenon of osteoinduction by biomaterials *in vitro*.

In conclusion, in our opinion, the *in vitro* assays which are nowadays used to study the potential performance of biomaterials *in vivo*, have a largely limited predictive value. It should be emphasized again that the existing *in vitro* assays have originally been designed to test the influence of growth factors, cytokines and hormones on the behavior of cells and organs. In these *in vitro* assays, the presence of a material has never been taken into account. However, in the studies involving biomaterials, there is, in addition to the material-cell interaction, which is supposed to be studied, often a material-medium interaction, which can be of high importance for the results and should therefore not be ignored. Prior to starting an experiment, the following questions should be answered: (i) is there an interaction between the testing material and the medium?, (ii) does this

interaction have a consequence for the results of the study? and (iii) is a similar interaction expected *in vivo*? If the biomaterial-cell culture medium seems to be an artifact of the system used, this effect of biomaterial-medium interaction should be removed. Knabe and coworkers suggested for example preincubation of the material in the medium prior to the start of cell culture and daily medium replenishment [8]. Although possibly successful for some biomaterials, this solution might be expensive, in particular if the cell culture is performed in presence of e.g. growth factors. Another possible solution could be the use of bioreactors in *in vitro* systems, with continuous monitoring and adjustment of the changing contents of the medium. Only if cells grow in the same medium, their interactions with different biomaterials can be compared in a useful way, and only then some careful conclusions can be drawn regarding their potential *in vivo* performance.

As mentioned previously, the choice of cells and assays can be of great importance on the outcomes of *in vitro* studies. This is important when e.g. osteoconductive potential of a biomaterial is studied. Instead of using mature osteoblasts, which are responsible for appositional bone growth rather than for *de novo* bone formation *in vivo*, the use of inducible and determined osteoprogenitor cells, as present in the bone marrow, might be more useful. When studying not yet unraveled complex biological phenomena such as osteoinduction, initially a pluripotent cell line should be used. The use of a homogeneous cell population can give an insight into processes governing osteoinduction. In the next step, adult mesenchymal stem cells from the recipient site (mostly muscle, or perivascular cells) should be used, as they are most probably involved in the process of osteoinduction.

22.4. *IN VIVO* MODELS FOR ASSAYING SYNTHETIC BONE GRAFT SUBSTITUTES

As described previously, the existing *in vitro* models used to assay safety and biological performances of synthetic bone graft substitutes are often not predictable for the *in vivo* situation, and therefore, every bone repair strategy needs to be established in an animal model before being used in human patients. Similar to the question whether *in vitro* models are predictive for the performance of biomaterials *in vivo*, it is important to investigate whether assays in animal models are predictive for the clinical setting. The number of publications in which the performance of synthetic bone graft substitutes and tissue engineered constructs for bone repair in humans is directly linked to the preclinical *in vivo* results is very limited. Therefore, this paragraph is limited to review of frequently used animal models for bone repair and regeneration. In addition, we address some limitations associated with the use of *in vivo* models.

22.4.1. Soft Tissue Models

As reviewed by Jansen [39], the first test following *in vitro* assays is *in vivo* compatibility of materials for short and prolonged periods of time. Soft tissue implantation is an attractive model to study safety of the materials in terms of e.g. toxicity and carcinogenicity, as it is rather inexpensive, readily available and yet relevant, as many materials used as bone graft substitutes come in contact with subcutaneous tissue, muscles, fasciae and tendons. The two most frequently used soft tissue models are

subcutaneous and intramuscular implantation. It has been shown that biocompatibility response of implant materials can differ between the two test sites, due to differences in vascularization, regenerative capacity and intrinsic stress. The selection of a suitable animal for biocompatibility testing is another complex issue. Mice, rats and rabbits are most often used for soft tissue implantations. The advantage of these, relatively small animals is their availability and low cost. However, their metabolic and wound healing properties differ from those of large animals and humans.

In addition to testing safety of biomaterials, soft tissue models are needed to study osteoinductive potential of DBM, purified BMPs or other cytokines and growth factors, osteoinductive properties of biomaterials and osteogenic properties of e.g. cells or tissue-engineered hybrids. An and Friedman gave an overview of the frequently used soft tissue models (e.g. subcutaneous, intramuscular, intraperitoneal and mesentery) to assay osteogenicity prior to orthotopic implantation [40].

Concerning the soft tissue models used to study the mechanisms of the, still largely unknown, phenomena such as osteoinduction, a careful choice of animal model and implantation site is of large importance. For example, as mentioned earlier, bone induction by BMPs is often observed in mice and rats, so these small animals are convenient for use in models to test osteoinductive capacity of DBM for example. Osteoinduction by biomaterials is, in contrast, rarely observed in small animals, so a large animal model is needed. However, it appears that there exist differences in osteoinductive potential of the materials implanted in different large animals; the same material induced more bone in dogs than in goats and rabbits [37], and even more bone was induced in baboons [41]. The reason for this interspecies difference is not completely understood yet, but it should certainly be taken into account when designing a study and interpreting its results.

In addition to the interspecies differences, large differences between individuals within species are often observed. For example, in addition to the difference in the response to BMPs between different animals [42], there are reports of differences in the response to BMPs between the individuals of the same species, probably due to genetic factors [43]. Similar differences were also observed in humans [44]. Also osteoinduction by biomaterials in goats has been shown to significantly differ between the individuals [16, 21, 45]. In order to avoid possible intraspecies differences, paired implantations, i.e. implantations of all test materials in all test animals are recommended.

22.4.2. Bone Fracture and Bone Defect Models

As reviewed by An et al. [46], diaphyseal fractures are most commonly used model to study bone fracture healing. Frequently used animals for studying diaphyseal fracture healing are rats, rabbits, dogs and sheep. Tibial fractures in rats, sheep and dogs are examples of the models of diaphyseal fractures. In addition to diaphysael fractures, epiphysometaphyseal osteotomy in rabbits, sheep, goats and dogs and delayed union and nonunion in rats, rabbits and dogs are other models to study bone fracture healing.

In order to study healing of bone defects, e.g. in the presence of a bone graft, four types of defect are typically used: calvarial-, long bone (or mandible) segmental-, partial cortical- (e.g. cortical window, wedge defect, or transcortical drill hole) and cancellous-bone defects. The commonly used animals are rats, rabbits, sheep and dogs, while goats and primates are sometimes used as well. The calvarial (critical-sized) defect and long

bone segmental defect are the most often used models for bone defect healing. Different animal models of bone defect repair are reviewed by An and Friedman [40].

22.4.3. Limitations of *In Vivo* Models for Assaying Bone Graft Substitutes and Recommendations for Their Improvement

Although animal models are used as a final test of the biomaterial performance prior to its use in the clinic, results of these preclinical studies might not be predictive for the materials performance in the clinical setting. In human patients, synthetic bone graft substitutes are used to repair and help regenerate (often large) defects caused by tumors, trauma, infections and hormonal disorders. Treatment of such diseased tissues in often elderly patients is hardly comparable with the treatment of an artificially made defect in the bone of young, healthy animals. In addition, it is conceivable that, based on frequently observed interspecies differences, results from any kind of animal are not (completely) predictive for the performance in humans. Finally, differences observed between individuals of the same species make it impossible to draw any general conclusions about the performance of a material as bone graft substitute.

Despite the fact that *in vivo* assays might not be completely predictive for the clinical performance of a material, their use can give valuable information about its biological behavior. It is, however, of great importance that *in vivo* studies are well designed and that their results are well analyzed. As already mentioned, paired implantations are important in order to exclude the effect of intraspecies variations as much as possible. In addition, more attention should be paid on finding non-invasive evaluation methods that allow for visualization of bone growth dynamics, as one of the important limitations of the classical *in vivo* studies is that only end results are visible. For example, use of fluorochrome markers is a helpful tool for the (qualitative or semi-quantitative) analysis of the bone growth dynamics [16, 47, 48]. The use of labeled cells is becoming helpful in studying the performance of bone tissue engineered hybrids [49]. Transgenic animals offer an important tool to study molecular pathways which take place in the process of bone formation in time [50]. However, so far, only transgenic mice and rats are available and therefore studying clinically relevant processes of bone formation remains a challenge.

22.5. BACKGROUND ON CARTILAGE TISSUE ENGINEERING

Tissue engineering approaches for repairing articular cartilage generally adopt two strategies; scaffold with cells, or scaffolds alone (Figure 22.4.). In general, scaffolds for cell-based strategies are intended to provide a compatible carrier for viable cells responsible for enhancing restoration of functional ECM and integration with surrounding native cartilage and subchondral bone. In strategies employing scaffolds alone, the scaffold material and geometry are designed to organize and enhance neo-tissue formation from host blood- and bone marrow-derived mesenchymal cells infiltrating the defect (i.e. osteochondral defects). The ability to further stimulate repair quality of cell-seeded scaffolds *in vitro* is possible by manipulating the culture environment via mechanical and/or bioactive stimuli, with accurate control offered via advanced bioreactor culture systems. Alternatively, scaffolds pre-seeded with genetically

modified cells, or scaffolds engineered to release bioactive or gene factors can be used to promote desired repair pathways or inhibit undesired processes.

In all cases, since articular cartilage is a load bearing tissue, it is important that the scaffold contain sufficient mechanical properties to protect cells and support maturation of engineered tissue [51, 52]. Balancing these mechanical requirements, it is preferable that scaffold biodegradation rate is controlled to allow suitable function of the implant in the short- to mid-term, but be completely degradable in the long term following repair tissue maturation.

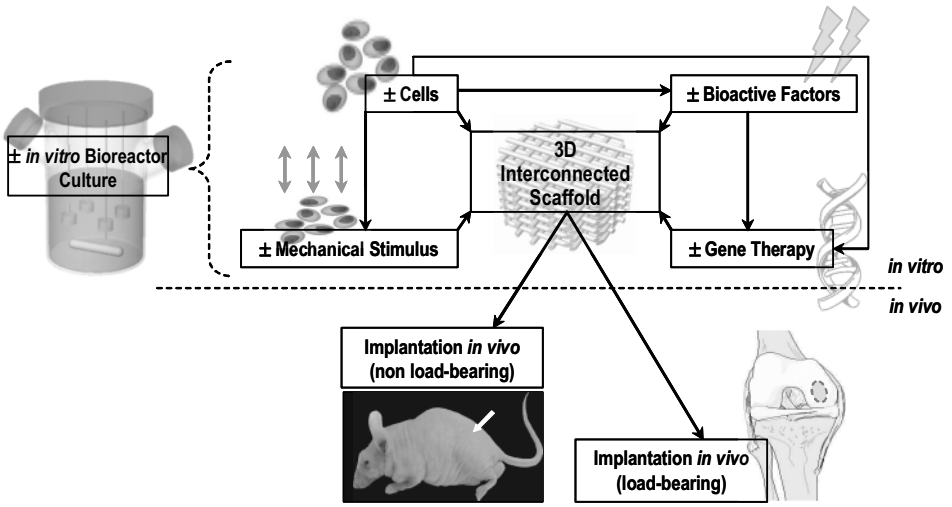

Figure 22.4. Diagram illustrating *in vitro* and *in vivo* model systems for evaluating cartilage tissue engineering strategies (adapted from Woodfield et al. [51]).

22.5.1. Scaffolds and Matrices

A full review of the various natural and synthetic biomaterials for cartilage tissue engineering strategies is beyond the scope of this chapter, and has been given previously [2, 51, 53, 54]. For the purpose of describing various *in vitro* and *in vivo* models systems and their limitations on scaffold design, it is important to introduce the common biomaterials and modern processing techniques used to develop porous 3D scaffolds for cartilage tissue engineering. These are summarized in Table 22.1.

By far the most common techniques for generating porosity include the various forms of foaming and particle leaching. Most of these techniques rely on generating porosity using porogens (*e.g.* a gas or particulate). While the size and, to a certain extent, the distribution of these porogens can be controlled during processing, their position and orientation to one another are inherently random. The control over scaffold architecture using these fabrication techniques are therefore highly *process* driven, and not *design* driven. This lack of control in pore structure, particularly with respect to the interconnectivity between pores causes considerable difficulties in designing porous scaffolds whose 3D pore architecture is critical for eliciting specific cell function and

Table 22.1. Scaffold materials and fabrication techniques for cartilage tissue engineering applications.

Biomaterial	Fabrication technique	Pore size (µm)	Application *in vitro* / *in vivo* [1]	Ref
Natural materials				
Collagen	Freeze drying, in situ cross-linked gel	-	Canine CH defect; rabbits OC defect; human OA defect	55-57
Chitosan	Thermosetting gel;	-	Chondrogenesis *in vitro* and in nude mouse; rabbit CH and OC defect	58, 59
Alginate	In situ cross-linked gel	-	Chondrogenesis *in vitro* and in nude mouse; rabbit OC defect	60-62
Agarose	In situ cross-linked gel	-	Chondrogenesis *in vitro;* rabbit CH defect.	63, 64
Hyaluronic acid (HYA)	Foaming + particulate leaching	26-83	Rabbit OC defect	65, 66
Synthetic polymers				
Polylactic acid (PLA)	Solvent casting + particulate leaching; non-woven fibre mesh	200-500	Chondrogenesis *in vitro* and in nude mouse; Rabbit OC defect	67-70
Polyglycolic acid (PGA)	Non-woven fibre mesh	<100	Chondrogenesis *in vitro* and in nude mouse; Rabbit CH defect	71-73
Polylactide-co-glycolide (PLGA)	Phase separation; solvent casting + particulate leaching	200-500	MSC Chondrogenesis in rabbit OC defect; Goat OC defect	68, 74
Polycaprolactone (PCL)	Fused deposition modelling*; nanofibre electrospinning	380-590 <10	MSC chondrogenesis *in vitro* and in subcutaneous rabbit model;	75 76
Polyehthylene glycol-terephthalate–polybutylene terephthalate (PEGT/PBT)	Compression moulding + particulate leaching; 3D plotting*	160-180 100-2000	Chondrogenesis *in vitro* and in nude mouse	77-80
Oligo-poly(ethylene glycol) fumarate (OPF)	In situ thermally cross-linked gel	-	Chondrogenesis *in vitro*	81
Poly(ethylene oxide)-dimethacrylate (PEG/DMA), polyvinyl alcohol (PVA)	In situ photo-polymerising gel	-	Chondrogenesis *in vitro* and in nude mouse	82, 83
Biphasic scaffolds (C: cartilage phase, B: bone phase)				
C: PLA B: hydroxyapatite (HA)	C: Solvent casting + particulate leaching, B: Indirect SFF (lost mould casting)*	C: 50-100 B: 300-800	Chondrogenesis, bone formation in nude mouse	84
C: OPF B: OPF	C + B: In situ thermally cross-linked gel	C: - B: -	Rabbit OC defect	85
C: PGA B: collagen/HA/tri-calcium phosphate (TCP)	C: non-woven mesh B: -	C: <100 B: -	Rabbit OC defect	86
C: PLGA/PLA B: PLGA/TCP	C + B: 3D printing* + particulate leaching	C: 250 B: > 125	Chondrogenesis *in vitro*	87
C: agarose B: devitalized bone	C: In situ cross-linked gel, B: machining of devitalized bone	C: - B: -	Chondrogenesis *in vitro* in anatomic scaffold	63

[1] *CH = chondral; OC = osteochondral; * = solid free-form fabrication (SFF) technique*

subsequent tissue formation. As a result, investigators have recently turned to solid free-form fabrication (SFF) techniques to produce porous scaffolds for tissue engineering applications [54, 75, 88-94]. SFF processing techniques allow highly complex and reproducible structures to be constructed one layer at a time via computer-aided design (CAD) models and computer-aided machining (CAM) processes. These techniques essentially allow researchers to *design-in* desired properties, such as porosity, interconnectivity and pore size, in a number of polymer and ceramic materials [90, 95-99].

These developments have opened the doors for more precise studies on the effects of designed pore architectures on cartilage tissue formation *in vitro* and *in vivo* [52]. The key breakthrough that these types of scaffold processing techniques offer is that they allow tissue engineers to more easily compare the influence of scaffold material, porosity and pore architecture on cartilage tissue formation *in vitro* and *in vivo*. They do so by allowing the pore geometry of the scaffold to remain fixed while maintaining a 100% interconnecting pore volume, without introducing any pores of random size or orientation, closed pores, or variation in material composition. Moreover, these techniques allow for designed scaffolds with enhanced control over mechanical properties [77, 79, 80].

22.5.2. Cells and Cell Sources

Cell types used in *in vitro* models for studying repair of both chondral and osteochondral defects have included committed chondrocytes [69, 71, 79, 100-102], cell-lines [103-108] or various progenitor, or mesenchymal stem cells (MSCs) from various sources [56, 109-113]. Articular chondrocytes are a common choice as these cells are responsible for maintenance and synthesis of essential cartilage matrix molecules. Highly promising alternative cell sources, which limit donor site morbidity associated with harvesting articular cartilage, include hyaline nasal septal cartilage [71, 114-117] and auricular cartilage [118]. Progenitor cell populations present within periosteum or perichondrium have also formed the basis for a number of *in vitro* studies [119-123]. Alternative stem cell sources receiving considerable attention for their chondrogenic potential are adipose-derived cells [124-127].

The main limitation of using committed chondrocytes is the difficulty in harvesting articular cartilage biopsies and limited number of cells that can be obtained from these small biopsies. Scaffold-based strategies to repair articular cartilage defects require large number of cells to generate sufficient volume of repair tissue, often necessitating the use of *in vitro* culture expansion techniques. One of the overriding limitations in cartilage tissue engineering is in overcoming the phenomenon of chondrocyte de-differentiation. Mature chondrocytes are well differentiated in their phenotype and are solely responsible for the maintenance of cartilage ECM components, characterized by the synthesis of predominantly type II collagen and the proteoglycan aggrecan [128]. When embedded within their native ECM, healthy chondrocytes exhibit a spherical morphology. However, when chondrocytes are released from their native ECM and cultured under conditions promoting a spread morphology, such as on 2D substrates, they progressively lose their original phenotype and display fibroblastic or pre-chondrogenic features, typically characterized by the expression of predominantly type I collagen and the proteoglycan versican [129]. This process is typically described as de-differentiation [130, 131], and can have considerable limitations for tissue engineering strategies resulting in inferior cartilage

tissue (i.e. fibro-cartilage) if suitable restoration of a differentiated chondrogenic phenotype cannot be achieved.

22.6. *IN VITRO* MODELS FOR CARTILAGE TISSUE ENGINEERING

In vitro assays are the cornerstone of any tissue engineer's toolbox for evaluating articular cartilage tissue formation and repair strategies. While numerous *in vitro* models exist, they all follow the general tissue engineering paradigm; combining cells, culture media, biomaterial substrates/scaffolds and various growth factors/cytokines.

22.6.1. Factors Influencing Chondrogenesis *In Vitro*

One of the challenges in tissue engineering is the design and fabrication of biodegradable scaffolds which influence specific cellular functions, and may thus regulate cell adhesion, proliferation, expression of a specific phenotype and extracellular matrix deposition in a predictable and controlled fashion. Chondrocyte re-differentiation (i.e. the post-expansion re-expression of chondrocyte phenotype) in scaffolds can be stimulated in a number of ways and are introduced briefly below.

22.6.1.1. Growth Factors/Cell Expansion

During the expansion phase, culture media supplemented with growth factors, such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), basic fibroblastic growth factor (bFGF) and platelet derived growth factor (PDGF) have been shown to influence chondrocyte proliferation rate [132], whilst enhancing the ability for subsequent cell re-differentiation [133-135].

The combination of these growth factors during expansion with other potent re-differentiation factors such as insulin, TGF-β and dexamethasone during 3D culture have been shown to stimulate cartilage tissue formation in scaffolds [71]. Cell expansion on 3D microcarriers, as opposed to 2D culture plates, has also been demonstrated to influence the downstream re-differentiation potential of chondrocytes [136-138]. Alternatively, cell free strategies rely of incorporation and controlled release of GF's, such as TGF-β and rhBMP-2 from biodegradable scaffolds to stimulate MSC differentiation and cartilage tissue formation.

22.6.1.2. Pellet/Mass Culture

Culture conditions, such as high-density pellet- or mass-culture techniques, which mimic cell condensation reactions associated with embryonic chondrogenesis or the *in situ* cartilage environment have also been shown to induce re-differentiation related to cell-cell and/or cell-matrix interactions [139-141]. These culture conditions are often used in combination with growth factor stimulation [142, 143].

22.6.1.3. Bioreactors/Mechanical Stimulation

Culture conditions which place tissue-engineered constructs in a dynamic fluid environment such as those present in spinner flask [144, 145] or rotating bioreactor culture [146-150], or which simulate *in situ* joint loading conditions via dynamic hydrostatic pressure [151-156] or mechanical compression [157-162], have also been suggested to stimulate chondrocyte re-differentiation [155, 156, 160, 163]. These dynamic culture conditions also aim at optimizing nutrient and waste exchange to engineered tissues. This is not only important for maintaining cell viability, but nutrient limitations themselves may also be involved in instructing cell function. For example, a low oxygen environment, comparable to conditions in native cartilage, has been suggested to be an instructive factor in promoting chondrocyte differentiation [164].

22.6.1.4. Cell-Scaffold Interactions

It has long been known that cell behavior on biomaterial substrates is related to both the physical and chemical properties of the substratum [165]. Several properties have been suggested as potential regulators of cell behavior including wettability, surface chemistry, equilibrium water content and roughness [165-167]. Furthermore, the specific substrate properties can either directly or indirectly effect cell adhesion, morphology and subsequent cellular activity by controlling adsorption of ions, proteins and other molecules from the culture medium [165, 168]. This is the scenario when seeding and culturing cells on biomaterials in serum-containing media, thereby exposing substrates to potent cell attachment proteins such as fibronectin (FN) and vitronectin (VN).

It is this molecularly populated surface that the cells sense and respond to biochemically by means of specific cell receptors such as the integrin family (*e.g.* $\alpha_5\beta_1$, $\alpha_V\beta_3$) via Arg-Gly-Asp (RGD) sequence domains [169-171], and CD44 via GAG-binding domains [172]. Therefore, the choice of biomaterial and the influence on protein adsorption and subsequent chondrocyte phenotype play a key role in promoting chondrogenesis *in vitro*.

22.6.1.5. 3D Scaffold Architecture

It is well established that chondrocytes require a 3D environment to maintain their differentiated phenotype and synthesize necessary ECM components such as collagen type II and GAG [51, 130, 133, 173, 174]. The influence of specific surface properties of various biomaterials on chondrocyte behavior has been so far mostly investigated using 2D films [175-179]. However, little is known about the specific influence of controlled changes in 3D scaffold architecture on chondrocyte (re)differentiation.

It has been suggested that scaffold architecture may control cell function by regulating diffusion of nutrients (*e.g.,* oxygen) and waste products, as well as influencing cell–cell interactions[180,181], e.g., engineered 3D fibre scaffolds containing a large, 100% interconnecting pore network have been shown to result in enhanced chondrocyte re-differentiation capacity and homogeneous distribution of cells and ECM compared to scaffolds with randomly generated and complex pore networks [79,80,180].

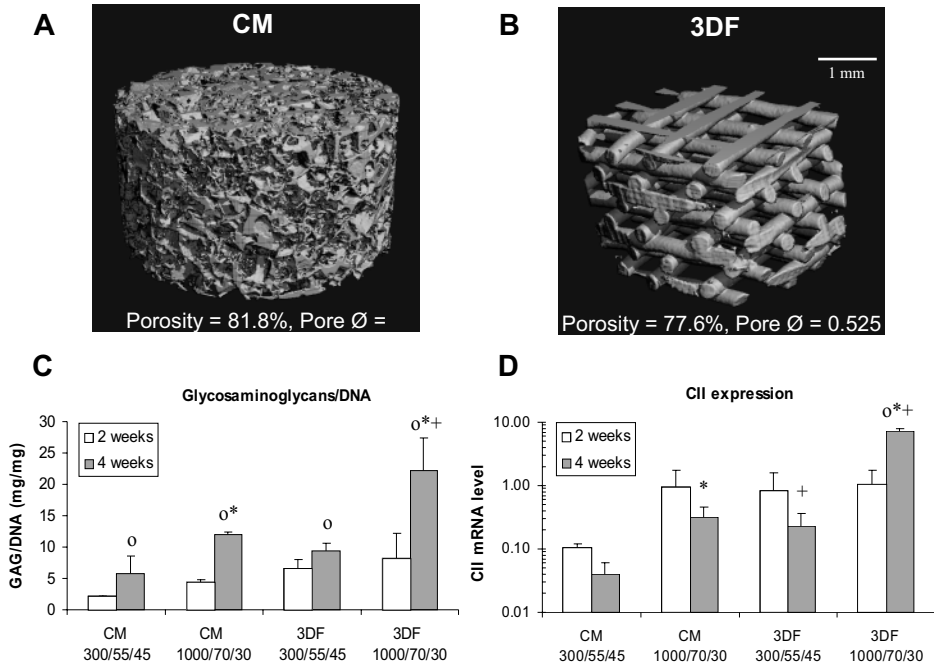

Fig 22.5. Micro-computed tomograpghy (μCT) images of porous PEGT/PBT polymer scaffolds with similar overall porosity, but varying pore architecture and average interconnecting pore diameter. (A) random pore architecture fabricated using compression molding (CM) and particle leaching techniques; (B) solid free-form fabrication (SFF) of a designed pore architecture in a layer-by-layer process via 3D fiber deposition (3DF). (C, D) The influence of PEGT/PBT scaffold composition (low PEG molecular weight 300/55/45 versus high PEG molecular weight 1000/70/30) and scaffold architecture (CM versus 3DF) on re-differentiation potential of expanded human nasal chondrocytes assessed via (C) GAG/DNA content, and (D) collagen type II mRNA expression. High PEG molecular weight composition (1000/70/30) in combination with a highly accessible pore volume and large diameter 100% interconnecting pore structure (3DF architecture) synergistically enhance restoration of human chondrocyte phenotype observed by significantly greater (GAG/DNA) and collagen type II mRNA at 4 weeks. Statistical significance ($p<0.05$) indicated by: ∗ = different from composition 300/55/45 for the same architecture; + = different from architecture CM for the same composition; O = different from 2 weeks of culture for the same architecture and composition (adapted from Miot et al.[77]).

Furthermore, when combining engineered 3D fiber scaffold architectures with a substrate promoting a chondrocyte phenotype, a synergistic increase in re-differentiation capacity of human expanded nasal chondrocytes in 3D fibre scaffolds has been observed [77] (Figure 22.5).

In an attempt to recreate the cartilage-bone interface and improve tissue integration in osteochondral defects, hybrid scaffolds and culture systems are being evaluated to tissue engineer both bone and cartilage layers. Such systems have been designed around a porous polymer or fibrin glue cartilage layer containing chondrocytes, anchored to a ceramic hydroxyapatite or calcium-phosphate base scaffold seeded either with or without bone progenitor cells [84, 86, 96, 182].

22.6.2. Limitations of *In Vitro* Models for Cartilage Tissue Engineering and Recommendations for Their Improvement

Pellet cultures are only useful in determining the (re)differentiation potential of chondrocytes without the influence of a biomaterial substrate [183]. While clearly advantageous for investigating the influence of various GF's and cell sources on chondrogenesis due to the small volumes of cells and culture media required, one limitation of this model is that localized chondrogenesis seen in small pellet cultures does not represent the culture environment in large 3D scaffolds of clinically relevant size, where nutrient diffusion and cell viability can vary greatly throughout the constructs [180]. Moreover, the absence of biomaterial-protein adsorption interactions from the culture medium, cell-biomaterial interactions and considerably reduced cell-cell interaction present in *in vitro* cultures on 3D scaffolds mean that to a large extent, positive results demonstrated in pellet cultures are not always directly transferable to 3D scaffolds *in vitro* [184].

Studies of chondrocytes or chondroprogenitor cells on 2D biomaterial substrates clearly offer the ability to study the influence of biomaterial-medium interaction via protein adsorption as well as cell-biomaterial and, to a certain extent, cell-cell interactions. However the influence of 3D architecture is neglected in such models. As mentioned previously, 3D architecture is vital for maintaining chondrocyte phenotype as evidenced in studies with chondrocytes maintained in 3D agarose or alginate gel culture and the prevalence for cell to typically undergo dedifferentiation when cultured on 2D substrates. The common perception is that events occurring in the 2D environment are not carried over when translated to a 3D environment. However, this may not exclusively be the case as a recent series of studies demonstrated that poly(ethylene glycol)-terephthalate – poly(butylene terephthalate) (PEGT/PBT) polymer substrates supporting maintenance of chondrocyte phenotype (i.e. a high collagen type II/I mRNA ratio) in expanded human nasal chondrocytes, also supported chondrogenic re-differentiation of these cells in identical culture conditions on 3D PEGT/PBT scaffolds produced using the same biomaterial composition [77, 185]. These data confirm that *in vitro* studies investigating controlled changes of substrate composition on chondrogenesis in 2D [185, 186] also translate to observations of substrate composition and architecture in 3D scaffolds [77].

When comparing various scaffolds for cartilage tissue engineering *in vitro*, a number of issues arise which limit the ability to draw direct comparisons between scaffolds, particularly in relation to the scaffold architecture and composition. Important constituents of a designed porous scaffold architecture include, but are not limited to, the following: porosity, pore interconnectivity (preferably 100%), accessible pore volume or permeability, pore size (i.e. size of pores and interconnection between pores), volume fraction (i.e. scaffold surface area to volume ratio), surface texture (i.e. rough micro-porosity or smooth), biomaterial composition, and scaffold degradation rate [51, 174]. Each of these factors together, or individually, can have an effect on cartilage tissue formation *in vitro*. For example, differences in pore architecture and volume fraction can influence the number and distribution of cells seeded within constructs [78]. Non homogeneous seeding can result in a high concentration of cells at the periphery of a scaffold, forming a fibrous capsule and preventing further cell migration and nutrient access, to the detriment of cells residing in the scaffold interior [148]. Subtle changes in scaffold composition may also influence protein adsorption and cell adhesion mechanisms resulting in altered proliferation and chondrocyte phenotype. Differences in pore interconnectivity and

permeability will affect nutrient and waste diffusion, such as oxygen [164, 180, 187], throughout scaffolds which ultimately will impact cell viability. Moreover, these nutrient gradients themselves can in turn have a large impact on cartilage tissue formation [180, 188].

Development of *in vitro* bioreactor cultures which provide medium flow and control over medium composition aim to enhance nutrient exchange and cell viability in large constructs of clinically relevant size [146, 164, 189, 190]. Yet few bioreactor systems take into consideration mechanical loading of the construct. Cartilage is an avascular, load bearing tissue, relying on mechanical compression and diffusion for nutrient and waste exchange with the synovium. Therefore, constructs engineered *in vitro* must be capable of supporting significant static and dynamic compressive stress comparable to native articular cartilage. *In vitro* bioreactor culture should include dynamic loading of constructs to evaluate construct longevity, cell differentiation, but most importantly, cell viability under physiologic stress [191, 192].

One further limitation of current *in vitro* models is that very few take into account the highly organized zonal structure of native articular cartilage in terms of cell distribution, GAG content and collagen type II orientation throughout the depth of the articular cartilage layer [51]. Recent *in vitro* studies have been aimed at recreating the zonal cartilage architecture by combining individual layers of chondrocytes isolated from superficial, middle and deep zone chondrocytes embedded in alginate or agarose gels [193, 194]. Other studies have engineered pore-size gradients into 3D scaffolds from which a heterogenous population of cells from all zones were seeded. These scaffolds promoted an inhomogeneous cell and ECM distribution similar to that seen in native cartilage [78]. However, it is unclear from studies to date, if it is possible to control the synthesis and zonal organization of collagen type II *in vitro*.

Each of these factors relating to scaffold architecture ultimately results in an altered differentiation state of the cell (e.g. GAG/DNA content) and the ability for it to synthesize cartilage ECM (e.g. collagen II) in the same quality and quantity as native articular cartilage. Many *in vitro* studies to date have compared various scaffolds *in vitro* where many of these factors are inherently different. While the conclusion may be that one scaffold performs better than another, it is often impossible to deduce if it was the influence of scaffold composition, accessible pore volume, or total cell content and distribution for example, without having precise control over the processing of scaffold architecture. Current efforts using SFF to produce designed scaffold architectures in which only one of the number of these factors is varied at a time are helping to unfold some of the key criteria that are necessary to engineer articular cartilage *in vitro* that can then be taken to the *in vivo* level [52, 77, 96, 97, 195].

22.7. *IN VIVO* MODELS FOR CARTILAGE TISSUE ENGINEERING

The ultimate success of any cartilage repair strategy must be established in animal models prior to clinical application. Such studies serve to highlight some of the existing problems confounding scaffold-based tissue engineering strategies in articular cartilage, as well as reveal some inherent limitations of the animal models themselves in relation to the clinical setting.

22.7.1. Non Load-Bearing Animal Models

As a first step in evaluating chondrogenic potential in an *in vivo* model, subcutaneous implantation of tissue engineered constructs in immuno-deficient mice can provide useful information in a non load-bearing environment, and help bridge the gap between *in vitro* and *in vivo* load-bearing models in larger animals [79, 80, 138, 196, 197]. Subcutaneous implantation of tissue engineered cartilage constructs typically results in enhanced tissue formation compared with constructs cultured *in vitro*, even in a controlled bioreactor environment. For example, Malda et al. [79, 180] demonstrated significantly higher GAG/DNA content and collagen type II staining in scaffolds subcutaneously implanted in nude mice after 4 weeks compared to constructs that remained in spinner flask culture *in vitro* over the same period [79, 180]. Implantation in subcutaneous pockets exposes constructs to host vasculature and local systemic growth hormones and the relatively inhospitable non-load bearing environment, however, there is the potential for host cells to infiltrate the scaffold and contribute to the repair process. Unless cells are tracked [198], limitations arise when evaluating MSC strategies for cartilage repair where it becomes unclear if the engineered construct and/or host MSC infiltration are responsible for the observed responses.

22.7.2. Load-Bearing Animal Models

Numerous animal models have been used to assess scaffold-based repair strategies in load bearing joints including rats, dogs, sheep and horses, however, most common small and large animal studies are carried out using rabbit [61, 64, 67, 68, 101, 199] and goat models [74, 200-204] respectively. While large animal models may more closely represent the human joint compared with small animal models, no animal model exists that is directly applicable to the human. Careful selection of animal age, chondral or osteochondral defect, partial or full load-bearing post surgery and uniform methods to assess outcome is necessary [205]. Immature animals (i.e. <6 months is the rabbit) may not be skeletally mature and have an increased spontaneous repair capacity which may override any repair strategy under evaluation.

22.7.3. Limitations of *In Vivo* Animal Models for Cartilage Tissue Engineering

As outlined recently by Hunziker [206], limitations in anatomical scale between osteochondral components in animals and humans are considerable, and relate to overall joint size, joint loading and thickness of and cell distribution within the cartilage layer itself. For example, the cartilage layer in the rabbit is only 200-300 μm thick compared to 3-5 mm thick in human cartilage [207].

The catabolic joint environment present in advanced degenerative diseases (e.g. in osteoarthritis, OA) as well as joint loading can also have significant consequences for scaffold-based repair with respect to the fate of implanted neo-tissue and scaffold degradation issues. These events are overlooked in *in vitro* studies and are difficult to assess *in vivo* in healthy animals. However, studies demonstrating clear differences in scaffold-based repair tissue between “freshly created” defects and “old” defects created 2

months prior to scaffold implantation, suggest the potential for future studies to incorporate a degenerative joint environment for evaluating cartilage repair [201].

The inexorable inconsistencies between animal models and the clinical setting make drawing definitive conclusions on various scaffold designs and repair strategies difficult. For example, recent studies have demonstrated the species variability in expansion and re-differentiation potential of human, dog and sheep chondrocytes [197]. It is clear from these studies that expansion and culture conditions optimized in animal models can by no means be directly translated to the clinical setting in humans. In addition, with the large variation in scaffold materials, cell types and culture conditions used, comparisons between *in vivo* studies are almost impossible. Standardized evaluation methods are necessary to not only compare different scaffold-based repair strategies, but also compare if such strategies are more favorable than traditional repair strategies, rather than just empty defects. Assessment criteria and histological grading scales such as that established by the International Cartilage Repair Society (ICRS) for example should become commonly adopted [208].

22.7.3.1. Scaffold Architecture

Many of the limitations discussed previously relating to the influence of scaffold architecture on *in vitro* culture also hold true for the *in vivo* environment. Issues of scaffold mechanical stability become more prevalent in the load bearing *in vivo* environment where a delicate balance between scaffold integrity and biodegradation rate is needed. In the case of osteochondral defects, scaffold architectures need to be designed to support integration with the subchondral bone and surrounding native cartilage. A range of biphasic constructs have been evaluated for this purpose and SFF techniques are leading the way in developing constructs based on polymeric and/or ceramic scaffolds with optimized architectures for cartilage and bone layers respectively [52, 84, 195]. Cell free strategies in which MSC's are recruited *in vivo* from the underlying subchondral bone spaces also require similar attention to scaffold architecture, as well as incorporating the controlled release of GFs (e.g. rhBMP-2) [209] or gene therapy products (e.g. cells over-expressing insulin-like growth factor) [61] necessary to stimulate both osteo- and chrondro-genic differentiation in various regions.

22.7.3.2. Cell Viability and Retention

Additional concern regarding scaffold-based repair strategies is the lack of knowledge with respect to the location, retention and viability of reparative cells once implanted *in vivo*. The large variation in repair results and limited success of tissue-engineered scaffold constructs to date may be, in part, related to the loss of cell viability and/or the inability to retain a critical number of chondrogenic cells in the proper region of the defect with time. For example, Ostrander et al. [210] seeded rabbit perichondrial cells in PLA constructs into osteochondral defects in rabbits. Repair tissue was harvested at various intervals from 0-28 days after implantation, and the number of donor cells determined via gender-specific gene tracking. Average cell viability was found to be 87% or more, with donor cells present in repair tissue for 28 days after implantation. However, the number of donor cells declined from approximately 1 million at time zero to

approximately 140,000 at day 28. This decline in donor cells was accompanied by a significant influx of host cells into the repair tissue. This is also significant for repair strategies that incorporate MSCs into osteochondral defects, as it cannot been excluded that enhanced tissue repair is derived from host cells recruited to the defect in response to the implant, rather than the repopulation of the tissue by the implanted MSCs. In this regard, Quintavalla et al. [202] implanted fluorescently labeled MSC/gelatin constructs into osteochondral defects in goats. The cells retained the dye up to 1 month and were detected by histology and flow cytometry. At intervals spanning 2 weeks post-implantation, gradual loss of implanted cells in the defect as well as fragments of gelatin sponge containing labelled MSCs in deep marrow spaces were observed. Although longer assessment times are necessary, the authors suggested that by determining the fate of implanted cells in short-term *in vivo* models, scaffold designs could be more rapidly optimized with respect to cell retention needed for successful, long-term cartilage regeneration. This was confirmed in a recent study where the length of pre-culture positively correlated with increased perichondral cell retention in tissue engineered PLA constructs following implantation in osteochondral defects in rabbits [67]. These results, however, act in direct contrast to other studies investigating the integration between the tissue engineered construct and the native surrounding tissue. Obradovic et al. [211] showed that integration in immature (1 week-old) *in vitro* cultured constructs with articular cartilage explants was enhanced due to cell proliferation and progressive matrix remodelling at the tissue interface, as opposed to mature (8 week-old) constructs. Obradovic et al., stated that while integration of immature tissue improved integration, the bulk mechanical properties of the tissue were low compared with mature constructs. This suggests that future scaffold designs which support rapid cartilage ECM synthesis and cell proliferation to enhance integration, but on the other hand, have sufficient mechanical stability to protect this newly-formed tissue from *in vivo* joint loads, could offer significant promise.

Unfortunately, since long-term functional stability of repair tissue *in vivo* is critical, the length of time needed to assess new treatment options, even at the pre-clinical stage, limits rapid innovation and development of scaffold-based repair strategies. Better *in vitro* models and more rapid *in situ* evaluation techniques, such as high resolution MRI [212] or imaging of luciferase markers in transgenic mice [213-215], that are capable of providing biochemical data (*e.g.* GAG) as well as structural and morphological images in real-time are necessary.

22.8. CONCLUSIONS

Today's *in vitro* assays in which biomaterials and tissue-engineered constructs for bone and cartilage repair and regeneration are tested often give inconclusive results and their predictive value for the *in vivo* performance is limited. One of the reasons for the limited predictive value on *in vitro* models is the undesired biomaterial-cell culture interaction. In addition, *in vitro* systems are often not representative for the *in vivo* situation in terms of cell population, nutrients supply, 3D environment and mechanical loading. Similarly, although they are a good source of valuable information about the biological performance of biomaterials and tissue engineered constructs, the results obtained from studies in *in vivo* models cannot directly be extrapolated to their performance clinically. The increasing number of new materials and technologies for

bone and cartilage regeneration requires fast and reliable *in vitro* and *in vivo* assays. However, the existing assays need improvements in order to be predictive for the final, clinical application of bone and cartilage repair strategies.

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