General discussion

GENERAL DISCUSSION

In diarthrodial joints, articular cartilage, calcified cartilage and subchondral bone form the osteochondral unit, intended as a functional unit that is uniquely integrated to ensure load transfer. During traumatic injuries functional and structural properties of the targeted joint tissue undergo considerable alterations, that will ultimately affect all the components of the osteochondral unit. The complex architecture of the knee joints, beside the very limited self-healing ability of articular cartilage, decreases the chance of complete regeneration. Currently, traumatic defects are repaired with microfracture, ACI and its derivates, autologous or allogeneic MSCs transplantation and osteochondral grafts [308]. Although these procedures have been used successfully and may slow down the onset of OA, it is well-known that pain often returns after an initial relief, due to inferior replication of hyaline cartilaginous tissue and partial or total failure of neo-tissue integration to the injury site [17]. Nevertheless, the use of cell-based therapies faces some disadvantages including the enormous costs for patients and health care systems, cell handling, safety and related regulatory issues [309]. This implicates that cell-free alternative strategies using biomaterials, need to be established to circumvent these impediments. The diarthrodial joints carry and distribute loads; hence, it appears to be inevitable to take also mechanical load into account for successful cartilage regeneration. Therefore, the evaluation of cell-free osteochondral regenerative approaches in combination with application of complex mechanical loading are of vital importance prior translation to clinical application. This thesis focuses on the development of a mechanically stimulated osteochondral defect model, which closely recapitulates an articulating joint microenvironment, to provide great advantages as preclinical screening tool for biomaterials and biomolecules. Furthermore, it evaluates the critical but challenging step of early endogenous cell recruitment into a hydrogel when subjected to joint movement.

Natural hyaluronic acid hydrogel as suitable candidate for cell-free cartilage repair approaches

Replacing damaged cartilage by using a 3D template hydrogel relies on important features that provide either adequate stiffness to resist repetitive loading or biochemical cues such as adhesive and chemotactic stimuli necessary for cell homing and directed cell migration. A key concept in regenerative medicine is the selection of a hydrogel that supports cell infiltration, proliferation, differentiation and guided tissue remodeling.

The hydrogel will form the cells› environment and should mimic the natural micro-architecture supporting cartilage repair. Instinctively, ECM has the potential to be the ideal hydrogel to provide the framework for tissue formation, therefore natural polymers which display inherent bioactivity are often favored as plausible biomaterials.

The properties of a hydrogel are established by the polymer chemistry from which it is synthesized [310]. GAGs are pivotal ECM building blocks used for joint lubrication and protein binding; the uniqueness of HA amongst this family is in the non-sulphated polymer backbone. The carboxylic acid present within the repeating units of glucuronic acid disaccharide confers very high negative charge densities ensuring the formation of HA gels with high swelling ratio and water content. HA can also strongly influence biomolecules, cell diffusion and differentiation by forming non-covalent bonds with cell-surface proteins and complexes (e.g. CD44) [311, 312], having a role in cartilage matrix stabilization. These interactions highly depend on its molecular weight and concentration. Also, an important factor of the HA scaffold bioactivity is its crosslinking density. In tissue engineered applications HA chemical crosslinking is typically required to reinforce its poor mechanical properties (due to high hydration and swelling) and reduce rapid degradation (due to the presence of hyaluronidase enzymes in native tissue) [313, 314]. Tunable hydrogel designs have enabled diverse studies including investigations of screening platforms for probing cell responses by dynamically stiffening or softening hydrogels to modulate cell phenotypic differentiation [315]. Taking advantage of HA hydrogel's tunable mechanics in a user-directed manner, we developed in **chapter 2** an advanced platform to screen biomaterials and biomolecules for their potential to support cell migration by using a 3D spheroid-based migration assay *in vitro* and an osteochondral defect model *in vivo.*

Cell spreading, migration and differentiation were found to be inversely proportional to the increase of HA-Tyr cross-linking degrees (150, 300 and 600 µM H_2O_2; **chapter 2**). Generally, the stiffer gels (highly crosslinked) have lower mesh size and lower cell flux throughout the matrix. When FB/HA hydrogel was used, hMSCs migrated much more in comparison to HA-Tyr hydrogels, in *in vitro* assays. Upon subcutaneous implantation by using an osteochondral model, this effect was even stronger as assessed by toluidine blue and collagen type II staining after 4 weeks.

Despite the similar stiffness (G') of FB/HA and HA-Tyr 150 and similar molecular weight of HA, it is likely that different HA concentrations and polymer backbone modifications drastically affected cell-material interactions, thereby influencing either cell adhesion and migration or differentiation among HA-Tyr and FB/HA hydrogels.

Beyond biophysical properties, hydrogels that more accurately recreate the complex milieus found in tissues include spatial patterning of numerous biochemical signals introduced to deliver factors to control cell response [316]. The encapsulation of PDGF-BB into HA-based hydrogels and its release described in **chapter 2**, chosen as the most potent chemoattractant after screening, differs among different HA-Tyr crosslinking densities and FB/HA gels. This has caused different exposure of hMSCs to the factor and in turn different migration responses *in vitro*. When applied *in vivo*, although the migration profiles of both HA hydrogels reflected the *in vitro* assay, the presence of the

factor did not increase cell infiltration in the osteochondral defect model. Moreover, it impaired chondrogenic differentiation in FB/HA gels. It is possible that the presence of PDGF-BB in those gels at this concentration and at this stage of remodeling stimulated an environment that prevents GAG deposition**.** Although the bioactive factor was not tested during chondrogenesis *in vitro* and a study had shown that the effect of PDGF-BB *in vitro* is to enhance matrix production [317], it is possible that when implanted *in vivo* the biomolecule is exposed to a multitude of different stimuli, may undergo exchange with its surroundings, may be washed out, recruited or may attract different cells from the neighboring tissues. This can explain the diminished chondrogenic differentiation observed in **chapter 2** in FB/HA gels containing PDGF-BB in osteochondral models after *in vivo* transplantation**.** These findings suggest that the factor may be important for endogenous MSC recruitment but may also impair chondrocytes differentiation, necessary for proper cartilage matrix reconstruction. Further studies will be needed to evaluate the dose-dependent effect of PDGF-BB on migration, differentiation and cartilage repair after *in vivo* transplantation.

Despite recent advancement in polymer design there is no single hydrogel that is ideal in every tissue engineering scenario. For instance, a hydrogel used as scaffold replacement for bone growth should be different from cartilage that does not require any blood vessel for growth. Many different biomaterials or bi-tri layer scaffolds are being developed by additive manufacturing to mimic different micro-architecture of cartilage [318], but the tissue complexity sets high standards to hydrogel design. In **chapter 2** the concept to evaluate biomaterials and biomolecules with enhanced endogenous recruitment and cartilage matrix formation was introduced by developing an advanced testing platform *in vitro.* It suggests that stiffer materials represent an obstacle to endogenous healing, thereby limiting matrix accumulation and neo-tissue integration, providing a further gel pre-selection prior to animal transplantation. By adjusting the mechanical properties of the hydrogel and perhaps varying polymer concentrations and crosslinking densities [50] or introducing porogens as sacrificial material (salt particle, fibers) with high degradation rate [319], stiffness can be modulated and porosity can increase to encourage cell infiltration, support differentiation and formation of either cartilage or bone tissue. Clinical translation needs extensive proof of functionality and safety, hence animal studies are a prerogative. To validate our *in vitro* findings, we have used ectopic implantation of osteochondral explants as a model for osteochondral defect repair to study early events of cell infiltration separated from influence of the joint host. A further investigation of the mechanical properties of the newly formed cartilage in an orthotopic defect model, where articular cartilage is subjected to proper mechanical stimuli, is surely of importance to assess long-term construct stability.

Mechanical stimulation of the selected matrix as an inevitable step for successful *in vitro* cartilage regeneration

The role of mechanical forces during development is well known [320] and physical stimuli are now considered as crucial as chemical factors in modulating cell fate and influencing tissue development. Researchers have hypothesized that a structure–function relationship influenced by dynamic loading could be the success for *in vitro* articular cartilage regeneration. To test these speculations the use of bioreactor systems experienced a rapid spread in the field of cartilage tissue engineering, to more closely mimic the mechanical stimuli experienced by chondrocytes within the joint. The responses of chondrocytes and MSCs to mechanical stimuli have been extensively reviewed by Johnstone *et al.* and Panadero *et al.* [321, 322]. They reported how changing input parameters, including hydrogel substrate, cells, growth factors, type of bioreactor and axes in combination with different loading regime, can dramatically influence the biochemical and biomechanical outcomes.

Generally, it has been shown that dynamic loading increases chondrogenic gene expression, proteoglycan content and biomechanical moduli. Mechanical compression applied to primary bovine chondrocytes seeded in agarose was found to enhance matrix production in a loading duration dependent manner [184]. A superior proteoglycan production was also observed when intermittent loading regime was applied to chondrocytes in comparison to unloaded controls [323]. Further contribution to these positive effects makes the pre-culture period of the construct followed by delayed loading; indeed it seems to be a prerogative [324, 325] that allows cells to build pericellular matrix necessary for effective mechano-transduction. However, the effect of the sole dynamic compression was not sufficient to produce tissue specific collagen. Therefore, the need to mimic the complex movement reminiscent more accurately of articular cartilage was considered to be a must. The supplementary application of dynamic uniaxial or multiaxial shear in combination with a compressive loading regime in chondrocytes cultured on ceramic surface or polyurethane scaffolds, pioneered by Waldman *et al.* and Grad *et al.* [80, 81, 219], confirmed the hypothesis that complex motion encourages the chondrocytic phenotype and the expression of genes associated with hyaline cartilage formation (COL2, ACAN, PRG4, COMP).

In analogy with the application of extrinsic mechanical loading, other reports have shown how diverse biomaterials can differently transmit mechanical signals to the embedded cell [138]. Based on biochemical data, results were not promising for cartilage tissue formation when bovine chondrocytes seeded in fibrin gels underwent mechanical stimuli; a reduced ECM deposition in comparison to unloaded controls showed an opposite trend to the agarose gel [326]. Moreover, the properties of both hydrogels are mutually different. **Chapter 3** emphasizes that a low intensity complex motion set-up promotes significant amount of total GAG when chondrocytes are

seeded into previously selected FB/HA hydrogels. The cellular response to load proved to be dependent on hydrogel substrates and highlights the importance of network crosslinking density and cell-matrix interaction, suggesting to some extend a positive effect of loading on matrix production when hyaluronic acid is conjugated with fibrin. This implies superior mechanical properties, longer term *in vitro* stability of gels containing encapsulated cells and an overall solid-elastic character (G'/G'') [60], which indicates higher mechano-resilience than fibrin hydrogels alone. Nevertheless, we could only apply very gentle loads, compared to e.g. fibrin-polyurethane scaffold; integrating cues that permit to recapitulate mechanical properties of the dense irregular cartilage tissue (synthetic polymers, such as aligned nanofibers of poly-(L-lactic acid), PLLA) in combination with e.g. cleavable linkage of natural ECM components to soften the matrix in presence of cells are hypothesized to be advantageous to find a good compromise to maintain a pro-regenerative environment.

Growth factors and chondroblasts, which act as mechanosensors, play a key role during the cartilage formation [327]. Many *in vitro* and *in vivo* studies with soluble factors are being performed without loading in order to enhance cell behavior, differentiation and tissue development [328] as potential way to ameliorate the properties of a hydrogel. However, little is known about the combinatorial mechanisms by which growth factors and loading elicit their effects on chondrocytes metabolism. In the works reported by Gigout and Eckstein, addition of the growth factor FGF-18 in gels, which is associated with mitogenic activity and increased ECM production, resulted in increased cartilage repair in both *in vitro* and *in vivo* models [165, 329]. The development of the N-terminal truncated variant FGF-18v increased the specificity for FGFR-3, the main receptor involved in chondrocyte differentiation and maturation [330]. Moreover, no studies have appreciated or reported the added effects of this factor in a tissue engineered construct within a dynamic loading environment. Therefore, we further complete the former research and conclude that intermitted and delayed biaxial motion in combination of FGF18v supplementation play a crucial role in cartilage development and maintenance of chondrocytic phenotype when seeded in FB/HA hydrogels (**Chapter 3**). The addition of FGF-18v alone was found to be insufficient to promote upregulation of cartilage matrix genes (e.g. ACAN, COMP, COL2, PRG4) in unloaded samples, conversely a marked increase was appreciated when dynamic loading was associated with the soluble factor. At tissue-level this effect was even stronger since safranin O positive repair tissue was formed by bovine chondrocytes in loaded FB/HA gels at FGF-18v concentrations tested, additionally confirmed by aggrecan and collagen type II production. These data suggest that differentiated chondrocytes can synergistically adapt to changing biochemical components and mechanical environment. In agreement with other literature [81, 198], mechanical stimulation in absence or presence of the factor downregulated matrix

metalloproteases involved in joint pathologies, thereby limiting ECM degradation and further proving the responsiveness of our gel platform under load.

The tendency of mature articular chondrocytes to undergo dedifferentiation when isolated and embedded in hydrogels is undesired for cartilage regeneration. Although COL1 and COL10 expression showed no significant differences between groups, it is worth noting that the ratio COL2/COL1 increased and COL10 expression markedly decreased after FB/HA hydrogel seeding. This suggests preservation of the differentiated state of chondrocytes within the 3D environment in loaded and unloaded conditions, likely preventing hypertrophy and in turn providing an environment that favors stable cartilage formation. Better insight into the newly formed cartilage matrix will provide valuable information; especially an accurate assessment of the cartilage tissue phenotype should include collagen protein quantification and differential proteoglycan and collagen type characterization, by quantitative methods such as hydroxyproline based collagen assay, PAGE, LC-MS and ELISA [331-333]. The density and degree of alignment of collagen fibers can be quantified by second harmonic generation images [334].

Longer culture time and total load duration would greatly favor increased collagen production and collagen fiber orientation, which is highly relevant for functional ECM formation important to fulfil the biomechanical role of articular cartilage in diarthrosis.

Taken together these data suggest the additive effect driven by the exogenous FGF-18v in a loaded FB/HA environment, which interdependently influence cellular metabolism by increasing the quality of the tissue engineered cartilaginous construct. Our finding also highlights the importance of the mechanical environment for cartilage tissue remodeling.

The effect of complex articulating motion on cell behaviour in an osteochondral environment

While a large body of literature has established that biomaterials, cells, biomolecules and loading regime are the main elements necessary to define tissue engineering within a dynamic loading environment, significant research is required in a more confined system to understand and predict hydrogel-cell construct behavior when surrounded by tissue during loading. Osteochondral *ex vivo* models [115] in which chondral and osteochondral defects can be generated, have been established with the view to study cartilage-bone interplay and select factors eliciting a healing process towards stable cartilage formation. However, these models have not considered the mechanical component as more predictable platform of the whole tissue response. Therefore, in **chapter 4** a mechanically stimulated *ex vivo* osteochondral defect model is presented and its response to complex motion pattern is validated using bovine chondrocytes embedded in a polyurethane-fibrin scaffold. Addition of compression and shear load provides the necessary improvement to enable more selective screening

of biomolecules and biomaterials for implants and evaluate possible constraints prior to moving to pre-clinical *in vivo* testing, thereby decreasing the number of animals to the essential. In line with previous findings [81, 219, 335], complex articulating motion promoted mechano-responsive articular surface protein and associated chondrogenic gene expression. The platform presented in chapter 4 possesses an advanced capacity of mechano-induced cartilage matrix gene expression due to a certain hydrostatic pressure buildup via external loading within the confined system. The addition of physiological joint kinematics also involves fluid pressurization, which has been shown to increase anabolic effects upon application to articular chondrocytes [336]. In polyurethane-fibrin scaffold these pressures would be imperceptible in an unconfined system because of high permeability of biomaterials.

It has become more and more evident that mechanical motion plays an essential role in the formation of new osteochondral tissues. Albeit the use of cell-laden scaffolds to certain extent might regenerate cartilage in defects after implantation, these approaches are still restricted by high costs, reduced cell sources, risks of disease transmission and complex manufacturing procedure [337]. Recent advancement tries to circumvent these drawbacks by developing acellular biomaterials that rely on endogenous cell recruitment to the wound sites offering great promise for *in situ* osteochondral regeneration. *In vivo* studies have monitored cell recruitment and defined different cell subpopulations involved in the migration process in a joint environment [338-344]. It is thought that providing an appropriate 3D template amenable for migration and differentiation, controlled and prolonged release of chemoattractant to enhance sufficient stem-progenitor cell recruitment to the injury site (**chapter 2**) and appropriate biochemical and biomechanical stimuli (**chapter 3**) are the key requirements for endogenous cartilage healing. However, it is still unknown how cells sense and respond to loads due to *in vivo* study limitations. Therefore, in **chapter 5** we employed the mechanically stimulated osteochondral defect model presented (**chapter 4**), filled with FB/HA hydrogel in presence or absence of PDGF-BB or SDF-1α gradients. This demonstrated that periodic mechanical stress inhibits early stages of endogenous cell infiltration.

Despite physical forces are important factors orchestrating the dynamic process of remodeling [307], it is likely that the early application of complex mechanical stimuli physically breaks down the newly synthesized matrix components, hence triggering an altered biological outcome. This effect is amplified in our hydrogel set up, where the biophysical characteristics are not sufficient to concurrently withstand load and attenuate tissue tension generated by the cell at early time point. However, we cannot exclude that the decreased cell migration number could be the result of a cell proliferation inhibition or an enhanced cell death [295]. . Upon loading cells colonizing the defect exhibit more spindle shape morphology, recalling a mesenchymal movement

[294] compared to the rounded and polygonal shape prevalent in unloaded samples, which could be more associated with ameboid migration phenotype [63]. This implies that mechanical input, besides hydrogel substrate and cell type, is also a determinant dictating the mode of migration.

The influence of external mechanical forces could dictate cell response in our system, however cell infiltration was still low and their uneven distribution may have limited strain-mediated chondrogenic differentiation and matrix remodeling, which might indicate that a well-orchestrated loading over time is crucial for successful endogenous cell remodeling. Obtaining homogeneous cell density and distribution within the hydrogel is a demanding but crucial step towards neo tissue formation [71, 345]. Similarly attracting the correct cell population (e.g. endogenous progenitor) while impeding immune cell infiltration should be a requirement for long-term implant survival. Interestingly, the provision of soluble chemical factors was not enough to augment cell chemotaxis and reverse the negative effect of loading on cell migration. Particular consideration should be given to determining and creating the right gradient concentration that would be effective and safe for enhancing cell mobilization. Another option could be to adapt hydrogel properties by reducing the volume/weight of HA in HA-Tyr, thereby reducing electrostatic interaction among their hydrophilic groups and the charged amino acid residues of the PDGF-BB, or by inserting degrading HA nanofibers to obtain a sustained fashion release and improve cell migration. Therefore, further testing in the model described is required.

The mechanically stimulated ex vivo osteochondral defect model has shown a potential route of cell migration in **chapter 5**, where cells either present in the subchondral bone or in the calcified cartilage cooperate to repair damage. In the context of the osteochondral model, the bone marrow and the contribution of bone lining cells, perivascular cells and cartilage progenitor cells cannot be ruled out, speculating that cross-talks among autocrine and paracrine factors secreted by different cell types may potentiate cellular activity. Nonetheless, the osteochondral defect model under load faces certain limitations. As it does not recapitulate the entire diarthrosis, it is not possible to replicate the whole range of events determining the body's healing response in cartilage repair *in vivo*. The wound healing process is significantly affected by the synovium and synovial fluid, which play a significant role in mesenchymal cell migration to the injury site [346], nutrient supply and metabolic by-product clearance, thereby influencing matrix production.

FUTURE DIRECTIONS

Although the research described in this thesis could not directly be translated into a clinical regenerative approach, important issues have been addressed for future developmental strategies. The selection of FB/HA hydrogel as ideal material to support endogenous cells to migrate into the wound site and trigger chondrogenic differentiation holds great promise for cell-free cartilage repair. A similar formulation of FB/HA hydrogel, containing higher molecular weight HA, is already approved as therapy for the treatment of osteoarthritis and associated pain. Longer *in vivo* studies should assess absence of hypertrophy and bone formation, which are the two most prominent drawbacks that impede stable cartilage formation. This limitation can be further circumvented by using specific pro-chondrogenic molecules such as antimir-221 [61], developing gene-activated matrices to orient progenitor cell fate *in situ* [347] or advances in genome engineering (CRISP-Cas9), which have made it easier to reprogram the intrinsic endogenous cell pathways by knocking down *MMP13* and enhancing collagen type II expression [348]. Despite this hydrogel presents a formidable physical assistance to cell ingress, resistance to high compressive load is critical. Reinforcement of the gel by combination of aligned fibers to promote organized matrix deposition [349], thereby inducing cell polarization and collagen alignment would lead to higher bulk mechanical properties while expediting cell infiltration.

Beside the identification of a multifaceted set of biochemical and biophysical factors with the aim to promote cell infiltration, modulating cell behavior to stimulate this process would be preferable. The entrapment of cargo molecules into the hydrogel is imperative to either attract the appropriate cells to colonize the defect or to enhance collagen deposition and bio-integration. However, no consensus on the ideal factor and its optimal dosage is yet available. A next step could be investigating the functional effects of different factors in a dose response dependent manner to increase cell density by prolonging the effect of the exogenous bioactive without inhibiting cartilage matrix deposition. At the same time, implant integration with the injured tissue could be promoted, for instance, by sequentially releasing collagenase and chemotactic factor in a coordinated and localized manner [350]. Methods to specifically label and track live cells over time are indispensable to define cell populations colonizing the defect, which would be beneficial for elucidating the underlying mechanisms of endogenous recruitment and regeneration. However, the imaging of the different cell types involved in joint defect restoration to distinguish each cell phenotype while differentiating remains a huge challenge.

At present, it is unclear how the mechanical loads affect endogenous reparative cells beneficial for neocartilage tissue formation, as *in vivo* studies are limited by the inability to monitor cell responses and determine the loads that the neo-tissue experience. In

this thesis the implementation of complex loading into osteochondral defect explants (**chapter 5**) shows temporal and spatial migration patterns in an osteochondral defect model, providing a perspective on how infiltrating cells transduce mechanical load into directed migration. This process is slowed down and negatively influenced by dynamic stimulation at early time point, highlighting a significant improvement in the understanding of osteochondral wound healing. Speculation remains on the timing at which the load induces the most beneficial response and whether hypertrophy can be prevented. While many diverse biomaterials are being developed to be tailored in a way to promote migration, differentiation and spatial organization, further testing for their suitability focusing on environmental factors that affect or potentiate cell infiltration under complex load is required. The underlying mechanisms governing the mechanical stress response need to be decoded and may offer new targets for therapies if novel pathways that exert its positive effects are discovered. The model described in this thesis provides an extensive platform that can be used for such screenings of materials in focal cartilage lesions to advance joint regeneration. The delicate balance between osteochondral remodeling and the application of mechanical loading was found to be vital in this highly dynamic process. No information is reported to date on the effectiveness of applying mechanical loading *in vivo* on cell-free implanted hydrogel with respect to cartilage and bone formation in an osteochondral site.

Since the healing of articular cartilage lesions in the knee depends on what happens in the period after surgery is performed, the model holds great promise to mimic surgeons' postoperative restrictions, recovery recommendations and rehabilitation protocols. The immediate post-operative care typically lasts 5-6 weeks (phase I) where the patient is non-weightbearing, while the phase II of rehabilitation (6-12 weeks) is marked by the progressive addition of weight bearing forces [351-353]. Time considered for the osteochondral explant maturation needs more attention, as it has been shown to play a critical role in tissue growth and integration [82]. To approximately resemble the rehabilitation practice, we have performed a preliminary experiment that showed that 5 weeks of explant pre-culture allowed high cell distribution and matrix deposition (**chapter 5**). Future research needs to clarify the effect of longer preculture time in the osteochondral explant before being subjected to mechanical load. Ideally, this model could provide useful insights to either prevent cartilage damage progression or to assess tissue remodeling in a controllable setting, so that mechanical modulation-based regeneration could be applied efficiently.

At the time the correct cells have colonized the defect, the success of long-term tissue regeneration is governed by the host immune response to the graft, since inflammatory cells invasion is often the prelude of graft-rejection or fibrotic tissue formation. With the view of translating a cell-free therapeutic approach, a mixed delivery of anabolic and inflammation modulating factors [53] could be more effective by e.g. promoting

spatial cell organization (gene delivery mediated or genome editing), while suppressing immune cell infiltration [354, 355]. The emerging field of immune-engineering seeks to promote tissue regeneration by modulating immune cells through engineering materials designed to release cytokines to drive macrophage polarization towards pro-healing M2 phenotype or present protein antigens conjugation to tune immune cell tolerance, memory and cytotoxicity [356, 357].

It is evident that the model under physiological stimuli has characteristics that only partially recapitulate the entire joint; the synovium and synovial fluid have also a significant influence on cell migration. Hence, mimicking the microenvironment by perhaps pre-exposing the explants to the synovium or synovium conditioned medium would be interesting to closely replicate the cascade of events that could lead to invasion of endogenous reparative cells into the lesion as it happens *in vivo*. An important component of joint kinematics, though less addressed in this thesis, is moving contact or rolling. These features of moving, accompanied by development of a cartilage-on-cartilage articulating motion system, would better resemble the joint niche and reduce friction of the loading system [233]. With the view of resembling traumatic defect as targeted tissue of the population intended for focal defect repair, the use of healthy human osteochondral explants could be an excellent step towards future clinical relevance, although the low donor availability and its high variability would pose a challenge. Nonetheless, the shortage of human explants could impede its clinical therapy, though currently there are no FDA approved engineered constructs grown in dynamic shear and compressive bioreactors. Considerations must be given in term of protocol standardization to entail high reproducibility and scalability

This thesis provided important biochemical and biophysical cues on hydrogel dynamics that resemble the optimal microenvironmental niche for directing osteochondral tissue growth and maintenance over time. The implementation of complex joint motion patterns should not be undervalued; the model described in this thesis can lead to a better understanding of the effects of mechanical stimuli on cell migration and differentiation in an osteochondral environment, and perhaps even boost, the natural biological cues to advance joint regeneration. This model demonstrates that *ex vivo* explants are new and interesting platforms that can help to bridge the still present low correlation between *in vitro* culture and *in vivo* biomaterial testing.