Use of environmental and physical stimuli in cartilage tissue engineering

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2014

This Ph.D. project was supported by the Dutch Programme for Tissue Engineering (DPTE)

Printing of this thesis was financially supported by:

Cover and Lay-out: Joost Das and Ruud Das
Printed by Ipskamp Drukkers

ISBN 978-94-6259-278-0

Use of Environmental and Physical Stimuli in Cartilage Tissue Engineering

Omgevings- en physische stimuli in kraakbeen tissue engineering

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 17 september 2014 om 11.30 uur

door

Rudolf Hendrikus Jozef Das
geboren te Valkenswaard

Promotiecommissie

Promotor:	Prof.dr.ir. H.H. Weinans
Overige leden:	Prof.dr. J.N.J. Philpsen Prof.dr. J.A.N. Verhaar Prof.dr. M. Karperien
Copromotor:	Dr. H. Jahr

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Chapter 1

General introduction

Articular cartilage

Articular cartilage is a smooth, white tissue that covers the ends of articulating bones. It enables them to move nearly friction-free, making joint movement smooth and painless. It also functions as a shock absorber, distributing load after impact to prevent damage to the underlying bone [1].

The tissue consists in essence of very few main components. Only one cell type maintains an extracellular matrix that consists in large part of just two structural proteins. It might, therefore, easily be assumed that damage to articular cartilage is readily repaired. However, damaged cartilage, resulting either from trauma or from a progressive disease like osteoarthritis, heals very poorly. Natural healing fails to reestablish the native organization of the extracellular matrix and surgical intervention has only limited success in long term follow up.

The relatively simple composition of articular cartilage, combined with a high prevalence of damage to the tissue, make it an attractive target for the development of cell based therapies for tissue repair. Unfortunately, the avascularity of the tissue, combined with its unique architecture, pose significant challenges for such therapies. The problem of creating large pieces of cartilage in vitro is especially daunting.

Composition of articular cartilage

Articular cartilage is an avascular, aneural and alymphatic tissue [2]. The wet weight of articular cartilage consist of only 20% of major structural proteins, the remaining 80% primarily being water and inorganic salts [3]. The dry weight of cartilage consists for more than 90% of only two extracellular matrix proteins, proteoglycan and collagen type II. The extracellular matrix that is comprised of these proteins is maintained by one cell type that represents a fraction (1-5%) of the wet weight of cartilage [4], the chondrocyte. Although the main constituents of articular cartilage are few, they belie a complex system with little self-renewing capacity.

Proteoglycans

Proteoglycan are a main part of articular cartilage, making up 5-10% of the wet weight of articular cartilage [3]. It consists of a core protein (aggrecan) to which numerous glycosaminoglycans (GAGs) are attached (figure 1). The core protein contains three globular domains (G1 and G2 at the N-terminus, and G3 at the C-terminus) [5]. Proteoglycans do not exist in isolation within the extracellular matrix, but as proteoglycan aggregates. These aggregates consist of a central filament of hyaluronic acid (HA) to which up to 100 aggrecan molecules are attached [6]. Attachment of aggrecans at the G1 domain to the central HA filament is stabilized by the presence of link proteins [7].

The central HA filament is a high molecular-mass GAG that is synthesized at the plasma membrane by hyaluronan synthases (HAS) [8]. Because HA is synthesized at the plasma membrane, it is directly extruded in the pericellular region. It is, therefore, likely that proteoglycan aggregate formation occurs initially in this region [6]. In humans, three isoforms of HAS (HAS1, HAS2 and HAS3) exist [9] that produce HA of differing chain lengths [10]. HAS3 produces the shortest, and HAS2 produces the longest chain lengths. In cartilage, primarily HAS2 and HAS3 mRNAs are expressed [11], while HAS1 appears especially abundant in synovial cells [12]. Within cartilage, HAS2 expression appears to be the highest and to be of crucial importance during embryonic development, as HAS2 knockout mice die mid-gestation [13]. However, the exact role of the different isoforms remains to be elucidated.

The GAGs attached to the core protein of aggrecan are highly negatively charged chondroitin sulfate (CS) and keratan sulfate (KS). Each core protein can be linked to approximately 100 CS and 50 KS chains [14]. The presence of such high amounts of polyanionic glycosaminoglycan chains results in a negative fixed charge density (FCD). This fixed negative charge attracts positive ions into the tissue. Consequently, water is attracted, resulting in swelling of the tissue.

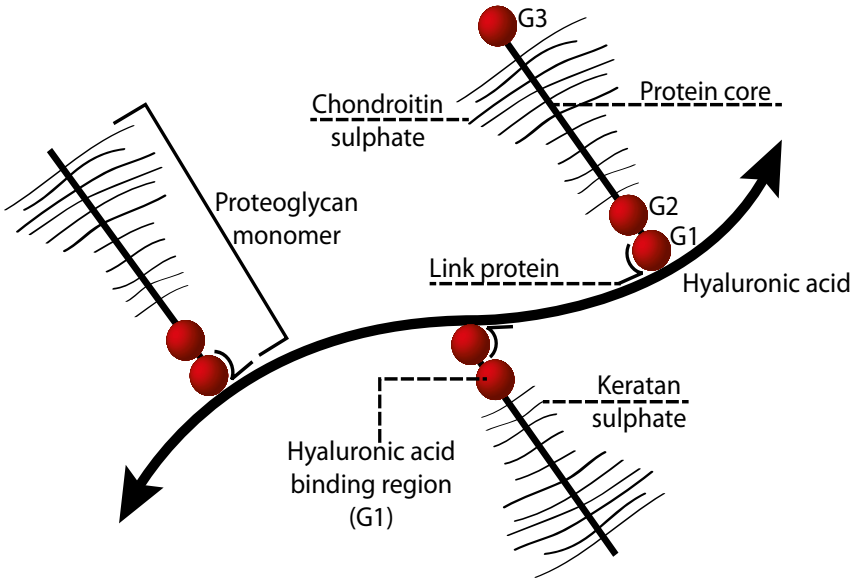

Figure 1: Schematic structure of the proteoglycan aggregate. The core protein of aggrecan is attached to the large hyaluronic acid filament at the G1 region by association with a link protein. Polyanionic sulfate groups radiate from the core protein. (http://www.ilo.org/safework_bookshelf/english?content&nd=857170059)

Collagens

The swelling caused by the attraction of water is limited by the major extracellular matrix protein in cartilage, collagen. Several types of collagen exist in articular cartilage, but type II is the predominant form, constituting 85-95% of the total collagen [15]. The other types include type III, V, VI, IX and XI, with type III, IX and XI making up the largest part of the remaining collagens [16]. In addition, collagen type X can be found in hypertrophic cartilage and the zone of calcified cartilage [17]. Every collagen molecule consist of three polypeptide chains (α-chains)

[18]. The composition of the α-chains depends on the type of collagen and can be either homotrimeric (three identical chains), as is the case for Collagen type II [19], or heterotrimeric (two identical α1 chains and a distinct α2 chain). The primary structure of the polypeptide is a repeat of three amino acids (Gly-Xaa-Yaa). Steric reasons impose that the first amino acid always is glycine (Gly), as any other amino acid would disrupt the helical conformation. In addition, a large part (>30%) of the Xaa and Yaa positions are filled by proline and hydroxyproline respectively. After post-translational modification (e.g. the hydroxilation of proline residues), the individual α-chains intertwine to form a triple helix reticulum [20]. This triple helix still contains large propeptides at both the N- and the C-termini that are cleaved after secretion into the extracellular space [21]. Cleaving of the propeptides reduces the water solubility of the collagen molecules and results in their aggregation into fibrils.

While several types of collagen exist in articular cartilage, it is a co-polymer of types II, IX and XI that forms the backbone polymeric template [16]. Collagen type IX can be covalently bound to both collagen type II and type IX itself [22]. Collagen type XI is primarily crosslinked with itself and it believed to limit lateral growth of the collagen type II fibril [23].

Chondrocytes

The only cell type present in articular cartilage is the chondrocyte (although reports on the presence of cells with multi-lineage potential, i.e. stem cells are also emerging [24, 25]). Chondrocytes are responsible for production of the extracellular matrix during growth and for maintenance of its integrity after growth arrest. To direct its function, chondrocytes respond to various external cues such as growth factors [26, 27], mechanical loading [28, 29] and cellular environment (e.g. oxygen tension) [30, 31].

Zonal distribution

Distribution of collagen, aggrecan and chondrocytes throughout the tissue is not homogeneous. Running from the articular surface to the subchondral bone, articular cartilage can be divided into four zones, i.e.

superficial, transitional, deep and calcified cartilage (figure 2) [32]. These zones differ distinctly in their orientation of collagen fibers and distribution of chondrocytes [33], as well as their aggrecan content [34]. In the superficial zone (10-20% of the cartilage thickness) the collagen fibrils are oriented parallel to the articular surface to provide high tensile strength to withstand stresses generated during joint loading. The transitional zone (20-70%) has a random organization of fibrils, while in the deep zone (70-100%) these fibrils are oriented perpendicular to the zone of calcified cartilage. This orientation results in the arch-like structure described in the early 20th century by Benninghoff [35].

While the collagen content does not vary significantly with depth, the amount of aggrecan does increase from the superficial to the deep zone. As a result the compressive modulus varies with depth [36] to provide a smooth transmission of loads.

Not only the composition of the extracellular matrix varies with depth, the content and morphology of chondrocytes is also depth-dependent. At the superficial zone the chondrocytes exhibit a flattened morphology, are clustered horizontally and are present at a higher density than in deeper zones [37, 38]. Chondrocytes of the superficial zone also produce proteoglycan-4 (PRG-4), a superficial zone protein believed to be involved in maintaining the lubrication properties of cartilage [39]. The middle zone is less densely populated with chondrocytes and they have a more spherical morphology. Finally, chondrocytes of the deep zone are larger and arranged in vertical columns.

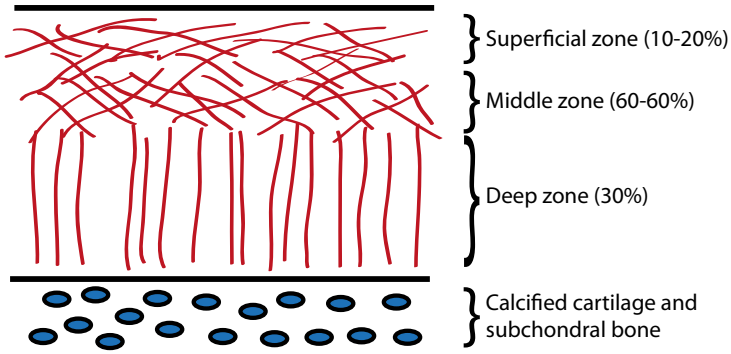

Figure 2: The zonal organization of articular cartilage

Cellular environment

Due to its avascularity, chondrocytes have to derive nutrients and oxygen primarily from the synovial fluid through diffusion [40], although recent studies also suggest a role for the subchondral bone [41]. As a result, chondrocytes experience oxygen tensions that are far below those experienced by most other cell types. In vivo, the pO2 of cartilage decreases from the superficial zone, where the pO2 is close to that of the synovial fluid (i.e. 10%), to the deep zone at the layer of calcified cartilage to nearly 1% [42-46]. While availability of oxygen is vital to cell survival, chondrocytes are well adapted to execute their function with minimal amounts of oxygen. For example, chondrocytes primarily employ anaerobic metabolism for the generation of ATP [47, 48].

Under these anaerobic conditions, ATP production is limited to the energy equivalents that are generated by conversion of pyruvate to lactate. Lactate is an acid that lowers the pH of the pericellular environment. Removal of this waste product from the tissue is hindered by diffusion limitations. Consequently, the pH of the pericellular environment is more acidic than that of other tissues. The pH of osteoarthritic cartilage can be as low as 5.5, as measure in grade 3 OA patients undergoing total hip replacement surgery [11953972] [50]. The presence of GAGs and expulsion of water by loading does not just cause an increase in [H+], but the tonicity (osmotic pressure) of the interstitial fluid on the whole is increased. In healthy cartilage, the tonicity ranges from 380 to 450 mOsm [51, 52]. In OA, the degeneration of the collagen network and the depletion

of GAGs results in a decrease in extracellular tonicity that depends on the stage of the disease. As a result, the tonicity of osteoarthritic cartilage can vary between 280 and 350 mOsm [51, 53]. Oxygen tension, together with extracellular pH and tonicity, forms a set of environmental stimuli that, through various pathways, can have a profound effect on cells that are exposed to them.

Joint loading not only causes a rise in extracellular pH and tonicity, it also creates a mechanical component that chondrocytes experience. This mechanical environment of chondrocytes creates fluid flows, (shear) stresses and cellular deformation. This deformation can be roughly 20% based on normal joint loading [54]. While this normal joint loading might be a prerequisite for healthy joint function [55, 56], excessive loading or normal loading of damaged cartilage can be detrimental and lead to osteoarthritis.

Osteoarthritis

The ability to move about freely without pain is a given for most people. The impact that pain during movement has on our quality of life becomes apparent even from such simple afflictions as a muscle ache. Osteoarthritis (OA) is a chronic disease that affects the articulating surfaces of joints, making movements painful and thereby severely impacting quality of life [57]. Over 10% of the population older than 60 suffers from OA to some degree [58] and this percentages increases to 60% for people aged 75 and up [59]. It is estimated this figure will only increase over the next years [60], since OA is primarily an age-related disease and the average age of the population steadily rises. This makes OA a highly relevant disease both socially (because of the loss of quality of life) and economically (because of loss of productivity). The economic burden in the Netherlands, for example, is estimated at almost 900 euro per patient per month in the working population [61].
Osteoarthritis is a multi-factorial disease, and includes inflammation of the synovium and changes to the subchondral bone, but it is characterized primarily by degradation of the articular cartilage.

Development of OA

The etiology of OA is complex and far from fully understood [62-64], but it is believed that an interplay of systemic, genetic, environmental and biomechanical factors contribute to the development of the disease. Also, trauma in early life such as meniscal tears, pattelar fractures and osteochondral lesions can lead to OA [65, 66]. Aggrecans are most likely the first constituent of cartilage to be affected by OA [3]. Aggrecan is progressively depleted concurrent with the severity of the disease. The initial loss is counteracted by increased synthesis, but production eventually fails to keep up with degradation, resulting in a net loss of matrix. In addition, OA is characterized by increased degradation of collagen type II. Collagenases, such as matrix metalloproteinases (MMP-)1 and -13, are responsible for the degradation, which results in irreversible damage to the matrix. The resultant weakened fibril network is no longer able to correctly withstand the swelling of the tissue and increased hydration ensues.

Current treatments for OA and cartilage defects

Several techniques exist to repair defects in articular cartilage, all of which are aimed at restoring function and reducing pain. Unfortunately, no technique exists yet that fully recovers the biomechanical properties of articular cartilage.

One of the simplest procedures available is arthroscopic lavage, usually combined with debridement. Aimed at reducing mechanical symptons and preventing further damage to articular surfaces, this technique has questionable benefits as results were not improved compared to a placebo procedure [67].

With drilling or microfracture the subchondral bone is penetrated to stimulate the formation of new articular cartilage [68, 69]. This relatively simple technique has excellent short term results [70-72] but the regenerated tissue is fibrocartilage with inferior biomechanical properties compared to native tissue. However, due to the simplicity and low costs of the procedure, microfracture is often the first choice for treatment of articular defects [73].

Osteochondral autografting (mosaicplasty) tries to circumvent the production of fibrocartilage associated with microfracture by transplanting full thickness osteochondral plugs from less-weight bearing regions of the knee joint to holes drilled in the defect site [74-76]. Although success has been reported [77] it still requires harvest from a healthy donor site, with the risk of associated donor site morbidity, and it is limited to relatively small defect sizes.

Autologous chondrocyte implantation (ACI), first described by Brittberg et al [78], uses an approach in which chondrocytes, isolated from a non-load bearing part of the articular cartilage, are expanded ex vivo then placed back in a defect and covered by a periosteal flap. Since its inception, the technique has been further developed and may now also include cells seeded on a three dimensional matrix. This matrix-induced autologous chondrocyte implantation (MACI) relies on the use of a biomaterial to provide extra mechanical integrity and load bearing capacity.

Tissue engineering

Tissue engineering refers to the part of science that aims to reconstruct ("engineer") tissues ex vivo for reimplantation [79]. In that light, the technique of (matrix-induced) autologous chondrocyte implantation can be considered a form of tissue engineering. Traditionally following the paradigm of autologous cells combined with a three-dimensional scaffold and growth factor stimulation, tissue engineering has since become vaster than the original concept. For one, the use of autologous cells that are native to the tissue (i.e. chondrocytes for cartilage) is no longer a given. Inching away from those cells, the use of cells with pluripotent capacity from other donor tissues, like bone-marrow, is getting increasingly important. But regardless the source, these cells have to be grown in a manner that ensures retention of, or differentiation into, a phenotype that is needed for tissue generation in vitro or in vivo. Secondly, the use of biomaterials to provide a three-dimensional structure has raised questions about the interaction of cells with their new environment. Lastly, the culture environment can be tailored in numerous ways that suit a specific cell type or application. Clearly, all permutations that arise from considering these

aspects of tissue culture make for a vast and interwoven field comprised of different areas of expertise.

Challenges in cartilage tissue engineering

The limited availability of autologous chondrocytes suitable for reimplantation is the reason these cells need to be expanded in vitro to obtain sufficient cell numbers. Expansion of chondrocytes is one of the main reasons for inferior tissue engineered cartilage constructs as it causes the cells to express progressively less of the matrix proteins that are specific for articular cartilage. Instead, they (de)differentiate towards a more fibroblast-like phenotype, losing their characteristic round morphology and expressing higher levels of the dedifferentiation markers; collagen type I and versican [80, 81].

As a result, production of cartilage specific extracellular matrix is severely hampered. Production of collagen type II is the major limitation, as GAG content can reach values comparable to native tissue [82-85]. Collagen type II, however, only reaches 15-35% of the native content [86-89] is specific studies. The limited production of collagen might have several causes. A dedifferentiated phenotype of expanded chondrocytes clearly causes a decrease, but even primary chondrocytes do not produce enough collagen type II. GAG production might impede collagen synthesis [90], the altered biochemical environment can affect the self-assembly of the collagen fibrils [91] while a change/reduction in mechanical stimulation can also reduce production or even cause fibril degeneration [92].

The lack of production of structural proteins also has implications for the mechanical functioning. While proteoglycan content can create sufficient compressive properties, the lack of collagen type II causes vastly inferior tensile properties. Use of biomaterials can aid in maintaining mechanical functioning, but full integration into the surrounding tissue is generally not successful.

Raw collagen content is not sufficient when trying to repair a tissue with such a specific architecture. The specific zonal organization of articular cartilage and the collagen fiber orientation within these zones contribute

massively to the mechanical properties of the tissue. Recapturing this organization in in-vitro culture will be especially difficult, although steps are being taken [93-95].

Bioreactors in tissue engineering

A common approach to maintain or regain the proper phenotype of expanded chondrocytes is it to try and mimic the natural environment of these cells as closely as possible. The term "bioreactor" in tissue engineering and biochemistry refers to a device that monitors and controls the culture process to support the growth of cells or tissues. For tissue engineering purposes, this usually means that a unique bioreactor is needed for every specific tissue type [96].

For cartilage, culture at physiological conditions includes maintaining cells at an oxygen tension and pH that approximates the physiological situation, seeding/embedding them in a three dimensional environment and applying physiological loading. Several attempts to mimic the natural environment, at least in part, have been described in literature [97-99]. However, this approach poses several issues. First of all, seeding cells in a three dimensional scaffold and maintaining viability for prolonged culture periods raises questions about mass transport of nutrients and waste products in 3D constructs [100]. Mechanical stimulation of the construct can aid in the transport of nutrients to deeper locations [101], but this stimulation should create stresses that aid in ECM production, not hamper it.

Finally, while the native environment of chondrocytes is well described, it is not certain that this environment is also the optimal environment for cartilage culture in vitro. Especially when tissue culture is started with expanded chondrocytes or stem cells, whose phenotype is very different from primary, adult chondrocytes, the optimal culture environment might differ significantly from the in vivo situation.

Bioreactors can also be employed to establish the best culture settings. The ability of bioreactors to tightly control a variety of parameters allows isolation of the effects of a single parameter on cells, or to evaluate the interplay between two or more parameters (e.g. hypoxia and pH). This

type of control is also required to identify the molecular pathways involved in these cultures.

Cell signaling pathways involved in environmental and physical stimulation

The control of the culture environment, made possible through the use of bioreactors, can be used to investigate the effect that different parameters have on cells. This use of bioreactors leads to better understanding of the mechanistic pathways that govern cell function under both normal and pathological conditions. Knowledge of the involvement of specific cell signaling pathways can lead to the development of targeted strategies.

It is clear that physical stimulation can alter cell signaling. For example, mechanical loading in the physiological (low) range of intensity have anti-inflammatory effects. This can lead to reduced levels of catabolic genes and increased expression of extracellular matrix components such as aggrecan and collagen type II [102].

Hypoxia-inducible factor 1α (HIF-1α) has been shown to be a key transcription factor for chondrocyte survival under hypoxic conditions [103], while HIF-2α appears to induce the chondrocyte phenotype through upregulation of SOX9 [104]. Hypoxia also has an effect of the pH homeostasis of chondrocytes [105], showing that the interplay of various control pathways is essential. The various effects of all these parameters needs to be elucidated to be able to devise a robust regenerative medicine strategy for cartilage repair.

Aim and outline of this thesis

As outlined in the general introduction, there are various ways to direct cell behavior by altering the physical environment. The challenging combination of mechanical stress and a hypoxic in the in vivo environment, makes the use of external physical stimulation especially interesting for articular chondrocytes. Various aspects of the use of external stimuli to study and manipulate chondrocytes in vitro are explored in this thesis.

In Chapter II the effect of mechanical stimulation on expanded chondrocytes is explored. The use of autologous chondrocytes, expanded in vitro to sufficient numbers for reimplantation, is standard in autologous chondrocyte implantation. These expanded chondrocytes express lower amounts of the chondrogenic markers collagen II and aggrecan, but they might also respond differently to the mechanical environment of the articular cartilage after reimplantation.

Mechanical stimulation is but one aspect of the physical environment of chondrocytes. The use of bioreactors for the control of the culture environment of cells is the topic of chapters III, IV and V. Chapter III describes a novel system for the control of culture parameters (e.g. oxygen tension and pH). These parameters directly influence several important pathways related to cell metabolism, extracellular matrix production and differentiation.

In Chapter IV the effects of bioreactor-controlled culture parameters (oxygen tension and pH) on chondrocyte redifferentiation are investigated. The mechanisms through which one of these parameters, hypoxia, exerts its effect on chondrocytes is still largely unknown. One of the most important growth factors involved in maintaining chondrogenic phenotype is Transforming Growth Factor-β (TGF-β). Its expression under hypoxia in relation to chondrocyte redifferentiation is investigated in this Chapter.

Chapter V explores the effects of an often overlooked culture parameter; the effect of osmolarity and the mechanism through which it regulates chondrocyte differentiation.

The potential of stem cells for regenerative medicine applications is clear, but myriad questions remain on the way cells can be conditioned or steered towards a specific phenotype so to better function after reimplantation in vivo. Hypoxic culture can be employed to pre-condition MSCs to enhance survival after introduction into a hostile environment such as infarcted heart tissue, it might enhance proliferation to speed up production for cell therapy and it can also direct differentiation in vitro. Chapter VI, therefore, reviews the various uses of hypoxic culture as a tool to manipulate MSC

behavior in culture and discusses the potential clinical implications.

In Chapter VII the various studies are discussed and results are extrapolated into a hypothesis on the proper use of physical stimuli with bioreactors to direct chondrocyte phenotype.

References

1. Huber, M., S. Trattnig, and F. Lintner, Anatomy, biochemistry, and physiology of articular cartilage. Invest Radiol, 2000. 35(10): p. 573-80.
2. Mankin, H.J., The response of articular cartilage to mechanical injury. J Bone Joint Surg Am, 1982. 64(3): p. 460-6.
3. Martel-Pelletier, J., et al., Cartilage in normal and osteoarthritis conditions. Best Pract Res Clin Rheumatol, 2008. 22(2): p. 351-84.
4. Muir, H., The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays, 1995. 17(12): p. 1039-48.
5. Doege, K.J., et al., Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. J Biol Chem, 1991. 266(2): p. 894-902.
6. Roughley, P.J., The structure and function of cartilage proteoglycans. Eur Cell Mater, 2006. 12: p. 92-101.
7. Morgelin, M., et al., Cartilage proteoglycans. Assembly with hyaluronate and link protein as studied by electron microscopy. Biochem J, 1988. 253(1): p. 175-85.
8. Weigel, P.H., V.C. Hascall, and M. Tammi, Hyaluronan synthases. J Biol Chem, 1997. 272(22): p. 13997-4000.
9. Spicer, A.P., J.S. Olson, and J.A. McDonald, Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. J Biol Chem, 1997. 272(14): p. 8957-61.
10. Itano, N., et al., Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J Biol Chem, 1999. 274(35): p. 25085-92.
11. Hiscock, D.R., B. Caterson, and C.R. Flannery, Expression of hyaluronan synthases in articular cartilage. Osteoarthritis Cartilage, 2000. 8(2): p. 120-6.
12. Recklies, A.D., et al., Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma cells. Biochem J, 2001. 354(Pt 1): p. 17-24.
13. Camenisch, T.D., et al., Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation

of epithelium to mesenchyme. J Clin Invest, 2000. 106(3): p. 349-60.
14. Kiani, C., et al., Structure and function of aggrecan. Cell Res, 2002. 12(1): p. 19-32.
15. Mankin HJ, M.V., Buckwalter JA, Iannotti JP, Ratcliffe A, Articular cartilage structure, composition, and function, in Orthopaedic Basic Science: Biology and Biomechanics of the Musculoskeletal System, E.T. Buckwalter JA, Simon SR, Editor. 2000, American Academy of Orthopaedic Surgeons Publishers: Rosemont, IL. p. 42-97.
16. Eyre, D.R., M.A. Weis, and J.J. Wu, Articular cartilage collagen: an irreplaceable framework? Eur Cell Mater, 2006. 12: p. 57-63.
17. Gannon, J.M., et al., Localization of type X collagen in canine growth plate and adult canine articular cartilage. J Orthop Res, 1991. 9(4): p. 485-94.
18. van der Rest, M. and R. Garrone, Collagen family of proteins. FASEB J, 1991. 5(13): p. 2814-23.
19. Miller, E.J. and V.J. Matukas, Chick cartilage collagen: a new type of alpha 1 chain not present in bone or skin of the species. Proc Natl Acad Sci U S A, 1969. 64(4): p. 1264-8.
20. Lamande, S.R. and J.F. Bateman, Procollagen folding and assembly: the role of endoplasmic reticulum enzymes and molecular chaperones. Semin Cell Dev Biol, 1999. 10(5): p. 455-64.
21. Prockop, D.J., A.L. Sieron, and S.W. Li, Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol, 1998. 16(7): p. 399-408.
22. Miles, C.A., et al., Differences between the thermal stabilities of the three triple-helical domains of type IX collagen. J Mol Biol, 1998. 277(1): p. 135-44.
23. Blaschke, U.K., et al., Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J Biol Chem, 2000. 275(14): p. 10370-8.
24. Williams, R., et al., Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage. PLoS One, 2010. 5(10): p. e13246.
25. Dowthwaite, G.P., et al., The surface of articular cartilage contains a progenitor cell population. J Cell Sci, 2004. 117(Pt 6): p. 889-97.
26. Guerne, P.A., A. Sublet, and M. Lotz, Growth factor responsiveness of

human articular chondrocytes: distinct profiles in primary chondrocytes, subcultured chondrocytes, and fibroblasts. J Cell Physiol, 1994. 158(3): p. 476-84.
27. van der Kraan, P.M., et al., Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage, 2002. 10(8): p. 631-7.
28. Grad, S., et al., Physical stimulation of chondrogenic cells in vitro: a review. Clin Orthop Relat Res, 2011. 469(10): p. 2764-72.
29. Smith, R.L., D.R. Carter, and D.J. Schurman, Pressure and shear differentially alter human articular chondrocyte metabolism: a review. Clin Orthop Relat Res, 2004(427 Suppl): p. S89-95.
30. Hirao, M., et al., Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. J Biol Chem, 2006. 281(41): p. 31079-92.
31. Murphy, C.L. and J.M. Polak, Control of human articular chondrocyte differentiation by reduced oxygen tension. J Cell Physiol, 2004. 199(3): p. 451-9.
32. Pearle, A.D., R.F. Warren, and S.A. Rodeo, Basic science of articular cartilage and osteoarthritis. Clin Sports Med, 2005. 24(1): p. 1-12.
33. Poole, A.R., et al., Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res, 2001(391 Suppl): p. S26-33.
34. Klein, T.J., et al., Depth-dependent biomechanical and biochemical properties of fetal, newborn, and tissue-engineered articular cartilage. J Biomech, 2007. 40(1): p. 182-90.
35. Benninghoff, A., Form und bau der Gelenknorpel in ihren Bezeihungen zur Funktion. Z Zellforsch Mikrosk Anat, 1925. 2: p. 783-825.
36. Schinagl, R.M., et al., Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. J Orthop Res, 1997. 15(4): p. 499-506.
37. Jadin, K.D., et al., Depth-varying density and organization of chondrocytes in immature and mature bovine articular cartilage assessed by 3d imaging and analysis. J Histochem Cytochem, 2005. 53(9): p. 1109-19.
38. Rolauffs, B., et al., Distinct horizontal patterns in the spatial organization of superficial zone chondrocytes of human joints. J Struct Biol, 2008.

162(2): p. 335-44.
39. Flannery, C.R., et al., Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and Is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. Biochem Biophys Res Commun, 1999. 254(3): p. 535-41.
40. McKibbin, B. and F.W. Holdsworth, The nutrition of immature joint cartilage in the lamb. J Bone Joint Surg Br, 1966. 48(4): p. 793-803.
41. Arkill, K.P. and C.P. Winlove, Solute transport in the deep and calcified zones of articular cartilage. Osteoarthritis Cartilage, 2008. 16(6): p. 708-14.
42. Falchuk, K.H., E.J. Goetzl, and J.P. Kulka, Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. Am J Med, 1970. 49(2): p. 223-31.
43. Lund-Olesen, K., Oxygen tension in synovial fluids. Arthritis Rheum, 1970. 13(6): p. 769-76.
44. Marcus, R.E., The effect of low oxygen concentration on growth, glycolysis, and sulfate incorporation by articular chondrocytes in monolayer culture. Arthritis Rheum, 1973. 16(5): p. 646-56.
45. Silver, I.A., Measurement of pH and ionic composition of pericellular sites. Philos Trans R Soc Lond B Biol Sci, 1975. 271(912): p. 261-72.
46. Treuhaft, P.S. and M.C. DJ, Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. Arthritis Rheum, 1971. 14(4): p. 475-84.
47. Grimshaw, M.J. and R.M. Mason, Bovine articular chondrocyte function in vitro depends upon oxygen tension. Osteoarthritis Cartilage, 2000. 8(5): p. 386-92.
48. Heywood, H.K., D.L. Bader, and D.A. Lee, Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration. J Cell Physiol, 2006. 206(2): p. 402-10.
49. Mow, V.C., C.C. Wang, and C.T. Hung, The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. Osteoarthritis Cartilage, 1999. 7(1): p. 41-58.
50. Kitano, T., et al., Biochemical changes associated with the symptomatic human intervertebral disk. Clin Orthop Relat Res, 1993(293): p. 372-7.
51. Maroudas, A.I., Balance between swelling pressure and collagen

tension in normal and degenerate cartilage. Nature, 1976. 260(5554): p. 808-9.
52. Urban, J.P., A.C. Hall, and K.A. Gehl, Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. J Cell Physiol, 1993. 154(2): p. 262-70.
53. Bank, R.A., et al., The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. Arthritis Rheum, 2000. 43(10): p. 2202-10.
54. Guilak, F., A. Ratcliffe, and V.C. Mow, Chondrocyte deformation and local tissue strain in articular cartilage: a confocal microscopy study. J Orthop Res, 1995. 13(3): p. 410-21.
55. Jurvelin, J., et al., Softening of canine articular cartilage after immobilization of the knee joint. Clin Orthop Relat Res, 1986(207): p. 246-52.
56. Vanwanseele, B., et al., Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement. Arthritis Rheum, 2002. 46(8): p. 2073-8.
57. Temenoff, J.S. and A.G. Mikos, Review: tissue engineering for regeneration of articular cartilage. Biomaterials, 2000. 21(5): p. 431-40.
58. Buckwalter, J.A., C. Saltzman, and T. Brown, The impact of osteoarthritis: implications for research. Clin Orthop Relat Res, 2004(427 Suppl): p. S6-15.
59. Oliveria, S.A., et al., Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. Arthritis Rheum, 1995. 38(8): p. 1134-41.
60. Brooks, P.M., The burden of musculoskeletal disease--a global perspective. Clin Rheumatol, 2006. 25(6): p. 778-81.
61. Hermans, J., et al., Productivity costs and medical costs among working patients with knee osteoarthritis. Arthritis Care Res (Hoboken), 2012.
62. Felson, D.T., et al., Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med, 2000. 133(8): p. 635-46.
63. Mandelbaum, B. and D. Waddell, Etiology and pathophysiology of osteoarthritis. Orthopedics, 2005. 28(2 Suppl): p. s207-14.
64. March, L.M. and H. Bagga, Epidemiology of osteoarthritis in Australia. Med J Aust, 2004. 180(5 Suppl): p. S6-10.

65. Buckwalter, J.A. and N.E. Lane, Athletics and osteoarthritis. Am J Sports Med, 1997. 25(6): p. 873-81.
66. Bader, D.L., D.M. Salter, and T.T. Chowdhury, Biomechanical influence of cartilage homeostasis in health and disease. Arthritis, 2011. 2011: p. 979032.
67. Moseley, J.B., et al., A controlled trial of arthroscopic surgery for osteoarthritis of the knee. N Engl J Med, 2002. 347(2): p. 81-8.
68. Insall, J., The Pridie debridement operation for osteoarthritis of the knee. Clin Orthop Relat Res, 1974(101): p. 61-7.
69. Steadman, J.R., W.G. Rodkey, and K.K. Briggs, Microfracture to treat full-thickness chondral defects: surgical technique, rehabilitation, and outcomes. J Knee Surg, 2002. 15(3): p. 170-6.
70. Miller, B.S., et al., Patient satisfaction and outcome after microfracture of the degenerative knee. J Knee Surg, 2004. 17(1): p. 13-7.
71. Passler, H.H., [Microfracture for treatment of cartilage detects]. Zentralbl Chir, 2000. 125(6): p. 500-4.
72. Steadman, J.R., et al., Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. Arthroscopy, 2003. 19(5): p. 477-84.
73. Yen, Y.M., et al., Treatment of osteoarthritis of the knee with microfracture and rehabilitation. Med Sci Sports Exerc, 2008. 40(2): p. 200-5.
74. Garrett, J.C., Osteochondral allografts for reconstruction of articular defects of the knee. Instr Course Lect, 1998. 47: p. 517-22.
75. Hangody, L., et al., Arthroscopic autogenous osteochondral mosaicplasty for the treatment of femoral condylar articular defects. A preliminary report. Knee Surg Sports Traumatol Arthrosc, 1997. 5(4): p. 262-7.
76. Hangody, L., L. Sukosd, and Z. Szabo, [Repair of cartilage defects. Technical aspects]. Rev Chir Orthop Reparatrice Appar Mot, 1999. 85(8): p. 846-57.
77. Marcacci, M., et al., Multiple osteochondral arthroscopic grafting (mosaicplasty) for cartilage defects of the knee: prospective study results at 2-year follow-up. Arthroscopy, 2005. 21(4): p. 462-70.
78. Brittberg, M., et al., Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med, 1994. 331(14): p. 889-95.

79. Bonassar, L.J. and C.A. Vacanti, Tissue engineering: the first decade and beyond. J Cell Biochem Suppl, 1998. 30-31: p. 297-303.
80. Schnabel, M., et al., Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage, 2002. 10(1): p. 62-70.
81. von der Mark, K., et al., Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature, 1977. 267(5611): p. 531-2.
82. Lima, E.G., et al., The effect of applied compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. Conf Proc IEEE Eng Med Biol Soc, 2006. 1: p. 779-82.
83. Lima, E.G., et al., The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. Osteoarthritis Cartilage, 2007. 15(9): p. 1025-33.
84. Elder, B.D. and K.A. Athanasiou, Effects of confinement on the mechanical properties of self-assembled articular cartilage constructs in the direction orthogonal to the confinement surface. J Orthop Res, 2008. 26(2): p. 238-46.
85. Waldman, S.D., et al., A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. Osteoarthritis Cartilage, 2006. 14(4): p. 323-30.
86. Kock, L., C.C. van Donkelaar, and K. Ito, Tissue engineering of functional articular cartilage: the current status. Cell Tissue Res, 2011.
87. Hu, J.C. and K.A. Athanasiou, The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs. Tissue Eng, 2006. 12(5): p. 1337-44.
88. Miot, S., et al., Cartilage tissue engineering by expanded goat articular chondrocytes. J Orthop Res, 2006. 24(5): p. 1078-85.
89. Eyrich, D., et al., In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. Tissue Eng, 2007. 13(9): p. 2207-18.
90. Asanbaeva, A., et al., Mechanisms of cartilage growth: modulation of balance between proteoglycan and collagen in vitro using chondroitinase ABC. Arthritis Rheum, 2007. 56(1): p. 188-98.
91. Saeidi, N., E.A. Sander, and J.W. Ruberti, Dynamic shear-influenced

collagen self-assembly. Biomaterials, 2009. 30(34): p. 6581-92.
92. Flynn, B.P., et al., Mechanical strain stabilizes reconstituted collagen fibrils against enzymatic degradation by mammalian collagenase matrix metalloproteinase 8 (MMP-8). PLoS One, 2010. 5(8): p. e12337.
93. Malda, J., et al., Localization of the potential zonal marker clusterin in native cartilage and in tissue-engineered constructs. Tissue Eng Part A, 2010. 16(3): p. 897-904.
94. Schuurman, W., et al., Zonal chondrocyte subpopulations reacquire zone-specific characteristics during in vitro redifferentiation. Am J Sports Med, 2009. 37 Suppl 1: p. 97S-104S.
95. Klein, T.J., et al., Tissue engineering of articular cartilage with biomimetic zones. Tissue Eng Part B Rev, 2009. 15(2): p. 143-57.
96. Ratcliffe, A. and L.E. Niklason, Bioreactors and bioprocessing for tissue engineering. Ann N Y Acad Sci, 2002. 961: p. 210-5.
97. Mauck, R.L., et al., Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng, 2000. 122(3): p. 252-60.
98. Zimmermann, W.H., et al., Tissue engineering of a differentiated cardiac muscle construct. Circ Res, 2002. 90(2): p. 223-30.
99. Mauck, R.L., et al., Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng, 2003. 9(4): p. 597-611.
100. Malda, J., et al., Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling. Biotechnol Bioeng, 2004. 86(1): p. 9-18.
101. Radisic, M., et al., Medium perfusion enables engineering of compact and contractile cardiac tissue. Am J Physiol Heart Circ Physiol, 2004. 286(2): p. H507-16.
102.Long, P., R. Gassner, and S. Agarwal, Tumor necrosis factor alpha-dependent proinflammatory gene induction is inhibited by cyclic tensile strain in articular chondrocytes in vitro. Arthritis Rheum, 2001. 44(10): p. 2311-9.
103. Schipani, E., et al., Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev, 2001. 15(21): p. 2865-76.
104. Lafont, J.E., S. Talma, and C.L. Murphy, Hypoxia-inducible factor

2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis Rheum, 2007. 56(10): p. 3297-306.
105. Milner, P.I., et al., The effect of O2 tension on pH homeostasis in equine articular chondrocytes. Arthritis Rheum, 2006. 54(11): p. 3523-32.

2

Chapter 2

In-vitro expansion affects the response of chondrocytes to mechanical stimulation

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Published as Das RHJ et al., In vitro expansion affects the response of chondrocytes to mechanical stimulation, Osteoarthritis Cartilage (2007)

Summary

Expansion of autologous chondrocytes is a common step in procedures for cartilage defect repair. Subsequent dedifferentiation can alter cellular response to mechanical loading, having major consequences for the cell's behavior in vivo after reimplantation. Therefore, we examined the response of primary and expanded human articular chondrocytes to mechanical loading.

Primary and expanded chondrocytes were stretched at either 0.5% or 3.0% at 0.5 Hz, 2 h per day, for 3 days. Gene expression levels of matrix components (aggrecan (AGC1), lubricin (PRG4), collagen type I (COL1), type II (COL2) and type X (COL10)) as well as matrix enzymes (matrix metalloproteinase 1 (MMP1), MMP3, MMP13) and SOX9 were compared to unstretched controls. To evaluate the effect of a chondrogenic environment on cellular response to stretch, redifferentiation medium was used on expanded cells.

In primary chondrocytes, stretch led to mild decreases in AGC1, COL1 and COL10 gene expression (maximum of 3.8-fold) and an up-regulation of PRG4 (2.0-fold). In expanded chondrocytes, expression was down-regulated for AGC1 (up to 21-fold), PRG4 (up to 5.0-fold), COL1 (10-fold) and COL2 (2.9-fold). Also, expression was up-regulated for MMP1 (20-fold) and MMP3 (up to 4-fold), while MMP13 was downregulated (2.8-fold). A chondrogenic environment appeared to temper effects of stretch.

Our results show that expansion alters the response of human chondrocytes to stretch. Expanded chondrocytes greatly decrease gene expression of matrix constituents and increase expression of MMPs, whereas primary chondrocytes hardly respond. Our data could be a reference for optimization of cell sources or expansion protocols for reimplanted chondrocytes.

Introduction

In autologous chondrocyte implantation (ACI) procedures, cartilage is harvested from an autologous donor site and isolated chondrocytes are expanded in vitro to obtain sufficient cell numbers before implantation into the defect site. However, during expansion culture, chondrocytes lose their specific chondrocytic phenotype and become more fibroblast-like [1, 2]. This phenotypical change, called dedifferentiation, is accompanied by a decreased gene expression of cartilage specific markers like collagen type II (COL2) [3]. This process might also alter the response of chondrocytes to extracellular stimuli. The current work studied the response of chondrocytes to mechanical stimulation after dedifferentiation resulting from monolayer expansion.

In their natural environment, chondrocytes are constantly deformed as a result of loading due to normal daily activities. Guilak et al.[4] estimated the loss of cell height of chondrocytes resulting from physiological loading to be approximately 20%. In vivo deformation will also occur in reimplanted chondrocytes after ACI. Normal physiological loading is generally regarded as a prerequisite for the maintenance of proper articular joint functioning, while injurious loading can lead to cartilage degeneration. Dynamic compression of bovine explants or three-dimensional scaffold cultures has indeed shown a stimulatory effect in vitro, not only on load bearing matrix components [5-11], but recently also on lubricin (PRG4) [12]. Other forms of mechanical stimulation like fluid flow induced shear stress [13, 14]and mechanical stretch [15, 16] also elicit a response in primary bovine chondrocytes.

In human normal, healthy chondrocytes Millward-Sadler et al.[17] found that cyclic stretch has an anabolic effect, as was shown by an increase in aggrecan (AGC1) expression and decrease in matrix metalloproteinase 3 (MMP3) expression. This effect was not seen in osteoarthritic (OA) chondrocytes, where no change in AGC1 or MMP gene expression was observed. This difference might be attributed to a change in mechanotransduction pathways between normal and OA chondrocytes [18-20]. In another study with human cartilage, Plumb and Aspden [21]

also showed that cyclic loading was not stimulatory in cartilage explants from human femoral heads. These results are contradictory to those found for young bovine chondrocytes, where loading was stimulatory [5-7]. Not only the source of chondrocytes determines the cell's response to mechanical loading. Wiseman et al.[22] showed that bovine articular chondrocytes in agarose constructs exhibited decreased proliferation and proteoglycan synthesis after monolayer expansion uponmechanical stimulation compared to primary chondrocytes.

Since expansion and the associated dedifferentiation of human chondrocytes is an essential step in ACI-like procedures, the effect of expansion on the matrixforming capacities warrants further investigation. Therefore, we investigated, through real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis, how human articular chondrocytes, after monolayer expansion, respond to stretch depending on their expansion and corresponding differentiation state. In addition, we examined whether a specific chondrogenic environment, which leads to redifferentiation to the chondrogenic phenotype, alters the response of expanded chondrocytes to stretch in terms of gene expression.

Materials and Methods

Cell culture

Cartilage was obtained from patients undergoing total knee replacement surgery (after approval by the local ethical committee; MEC2004-322). Full thickness cartilage was harvested, treated with 0.2% protease in physiological saline solution (Sigma, St. Louis, MO, USA) for 90 min and subsequently digested overnight in basal medium [Dulbecco's modified eagle medium (DMEM), 4.5 g/l glucose with 10% Fetal Calf Serum (FCS), 0.1% gentamicine and 0.6% fungizone (all Invitrogen, Scotland, UK)] supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a heamacytometer. The primary chondrocytes were then either seeded at a density of 7500 cells/cm2 in a T175 culture flask for expansion culture or seeded at a density of 300,000 cells/well in collagen type I

(COL1) coated Flexcell six-well plates (Flexercell, McKeesport, PA, USA). The cells plated for expansion were cultured for three passages. These expanded chondrocytes were then seeded at a density of 300,000 cells/ well in the Flexcell COL1 coated six-well plates (figure 1).

Mechanical stimulation

Cells were left to adhere firmly to the flexible membrane of the six-well plate during a 5 day pre-culture with basal medium. On day 5, cells were stretched using a modified Flexcell set-up (Flexercell, McKeesport, PA, USA) inside an incubator (37oC, 5% CO2). This set-up was previously described [23]. Briefly, a low pressure created under the six-well plates pulls the flexible membrane over a loading post, resulting in homogenous biaxial strain. The size of the loading post and the level of the pressure correlate to the amount of stretch applied to the adherent cells. Loading posts of 25 mm and 30 mm diameter were used, resulting in applied strains of 3.0% and 0.5%, respectively. Cyclic stretch at a frequency of 0.5 Hz was applied twice daily for 1 h with a 1 h rest period. This protocol was repeated for 3 days. Unstretched controls were placed in the device without stretching the membranes.

Redifferentiation medium

To examine the effects of a chondrogenic environment, experiments were also conducted with redifferentiation medium [2]. This medium consisted of DMEM high glucose, 1:100 insulin-transferrin-selenium A supplement (ITS) + (BD Biosciences, Bedford, MA, USA), 10 ng/ml transforming growth factor-b2 (TGF-b2) (recombinant human, R&D Systems, Abington, UK), 10 ng/ml insulin-like growth factor-1 (IGF-1), 25 mg/ml L-ascorbic acid 2-phosphate (both from Sigma, St. Louis, MO, USA), 0.1% gentamicine and 0.6% fungizone (both from Invitrogen, Scotland, UK). The redifferentiation medium was added at the onset of stretch.

PCR

Directly after the last stretch cycle total RNA was isolated using the Nucleospin II kit according to the manufacturer's instructions (Machery-Nagel, Düren, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop_ ND1000, Isogen Life Science, The Netherlands). For cDNA synthesis and real-time quantitative PCR (qPCR) methods see Uitterlinden et al.[24]. An ABI7000 was used for

Table 1
List of primers

Gene	Acc.No.	Primer	Nucleotide sequences
GAPDH	BC_083511	HsGAPD_F	ATGGGGAAGGTGAAGGTCG
		HsGAPD_R	TAAAAGCAGCCCTGGTGACC
		HsGAPD_FAM	CGCCCAATACGACCAAATCCGTTGAC
AGC1	NM_001135	HsAGC1_F	TCGAGGACAGCGAGGCC
		HsAGC1_R	TCGAGGGTGTAGCGTGTAGAGA
		HsAGC1_FAM	ATGGAACACGATGCCTTTCACCACGA
MMP1	NM_002421	HsMMP1_F	CTCAATTTCACTTCTGTTTTCTG
		HsMMP1_R	CATCTCTGTCGGCAAATTCGT
		HsMMP1_FAM	CACAACTGCCAAATGGGCTTGAAGC
MMP3	NM_002422	HsMMP3_F	TTTTGGCCATCTCTTCCTTCA
		HsMMP3_R	TGTGGATGCCTCTTGGGTATC
		HsMMP3_FAM	AACTTCATATGCGGCATCCACGCC
MMP13	NM_002427	HsMMP13_F	AAGGAGCATGGCGACTTCT
		HsMMP13_R	TGGCCCAGGAGGAAAAGC
		HsMMP13_FAM	CCCTCTGGCCTGCTGGCTCA
SOX9	NM_000346	HsSOX9_F	CAACGCCGAGCTCAGCA
		HsSOX9_R	TCCACGAAGGGCCGC
		HsSOX9_FAM	TGGGCAAGCTCTGGAGACTTCTGAACG
COL1	NM_000088	HsCOL1_F	CAGCCGCTTCACCTACAGC
		HsCOL1_R	TTTTGTATTCAATCACTGTCTTGCC
		HsCOL1_FAM	CCGGTGTGACTCGTGCAGCCATC
COL2	NM_033150	HsCOL2_F	GGCAATAGCAGGTTCACGTACA
	NM_001844	HsCOL2_R	CGATAACAGTCTTGCCCCACTT
		HsCOL2_FAM	CCGGTATGTTTCGTGCAGCCATCCT
COL10	NM_000493	HsCOL10_F	CAAGGCACCATCTCCAGGAA
		HsCOL10_R	AAAGGGTATTTGTGGCAGCATATT
		HsCOL10_FAM	TCCAGCACGCAGAATCCATCTGA
PRG4*	NM_005807	HsPRG4_F	TTGCGCAATGGGACATTAGTT
		HsPRG4_R	AGCTGGAGATGGTGGACTGAA
		-	-

***SYBRGreen I assay.**

cycling. Taqman™ or SybrGreen™ I assays were performed on AGC1, proteoglycan 4 (PRG4, alias lubricin or superficial zone protein), COL1, COL2 and COL10, MMP1, MMP3, MMP13 and transcription factor (sex determining regionY)-box 9 (SOX9). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. All primer and probe nucleotide sequences for gene amplifications are listed in Table 1.

Data analysis

Expression was normalized to GAPDH and expressed relatively using the 2-(ΔΔCt) method of Livak [25]. Subsequently, expression levels of unstretched control conditions were set to 1 and stretched conditions were plotted relative to controls. Results are means plus standard deviation. Statistical significance was determined using a KruskaleWallis test (SPSS Inc., Chicago, IL, USA) prior to testing stretched vs unstretched conditions by ManneWhitney test. Differences were considered significant when $P < 0.05$. For every experiment with primary cells, six control wells were

Table 2
Number of donors and technical repetitions for every experimental condition

Condition	Stretch level	n	Number of donors
Primary chondrocytes	Control	19	4
	0.5%	11	4
	3.0%	12	4
Expanded chondrocytes on basal medium	Control	12	3
	0.5%	9	3
	3.0%	9	3
Expanded chondrocytes on redifferentiation medium	Control	6	2
	0.5%	6	2
	3.0%	6	2

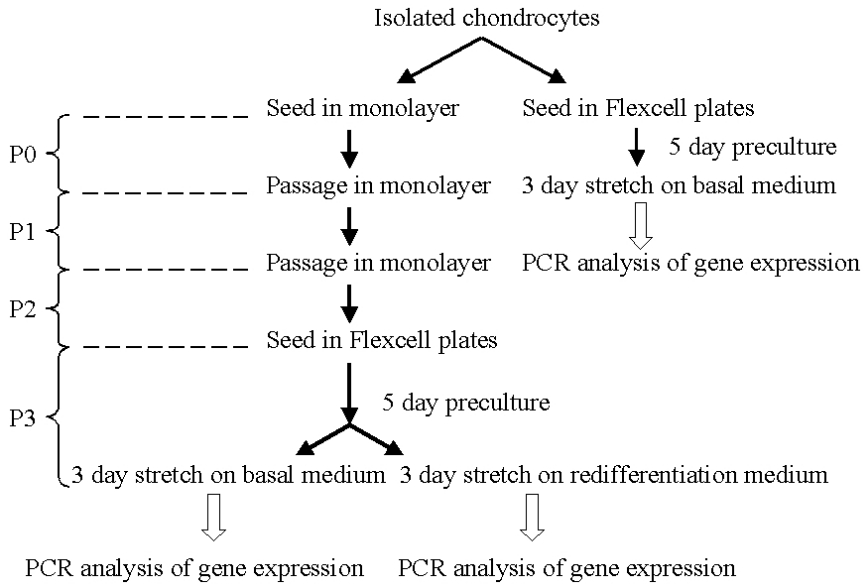

Figure 1: Layout of experimental set-up

used for each donor, while three wells were used for 0.5% and three wells for 3.0% strain. The first experiment with expanded cells had the same set-up as the experiments with primary cells. For the other experiments with expanded cells, three wells were used for unstretched controls on basal medium and three wells were used for unstretched controls with redifferentiation medium. For both stretched conditions (0.5% and 3.0%), three wells per plate were used with basal medium and three wells were used with redifferentiation medium. Table 2 summarizes experimental details: some wells were lost due to low cell yield after harvest.

Results

Effect of expansion culture on levels of gene expression

Upon expansion in monolayer culture, gene expression of COL1 was up-regulated while SOX9 expression was down-regulated, typical for dedifferentiation toward a more fibroblast-like phenotype (figure 2). At the same time, COL2 is hardly expressed and COL10 expression is completely absent in dedifferentiated chondrocytes, also consistent with the shift toward a fibroblast-like state. Also, expression levels of MMP1, MMP3 and MMP13 were considerably lower after expansion.

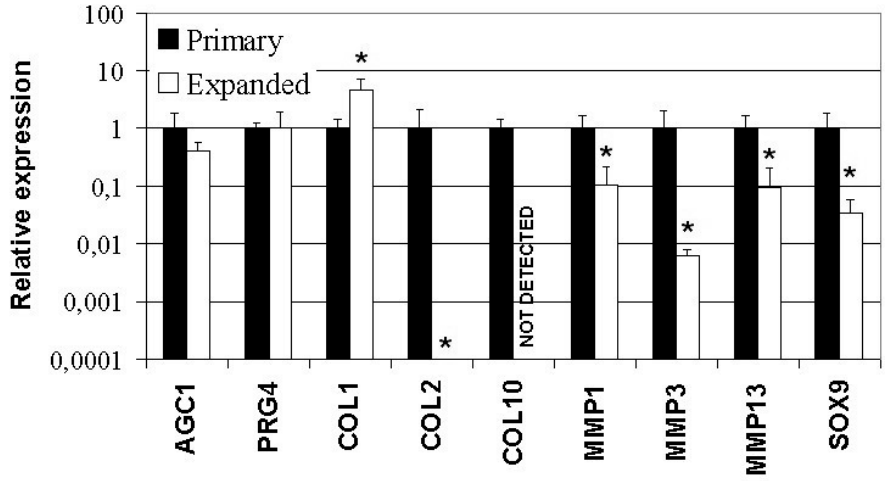

Figure 2: Relative gene expression levels of unstretched primary chondrocytes versus expanded (P3) chondrocytes. * indicates significant difference (P< 0.05).

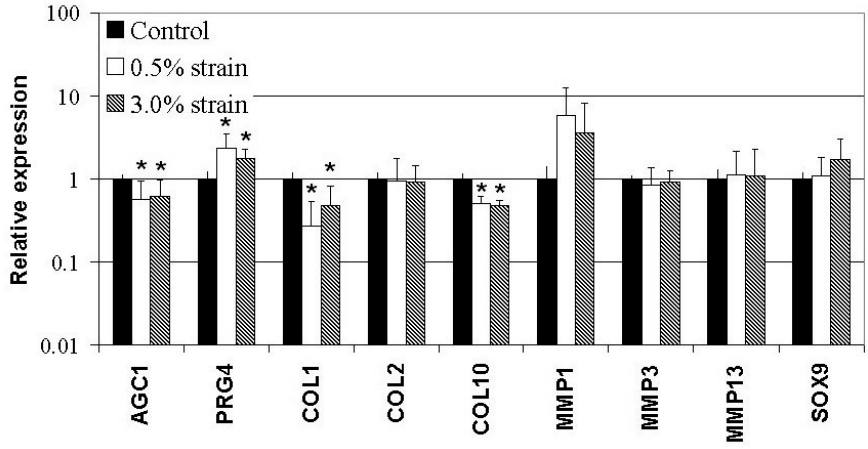

Figure 3: Gene expression of primary chondrocytes under strain (0.5% and 3.0%) relative to unstrained controls. * indicates statistically significant difference with control (P<0.05).

Effect of stretch on primary chondrocytes

Gene expression of matrix components (AGC1, PRG4, COL1, COL2 and COL10) was moderately altered by stretch in P0 chondrocytes (figure 3). AGC1 and COL1 were down-regulated in a response to mechanical

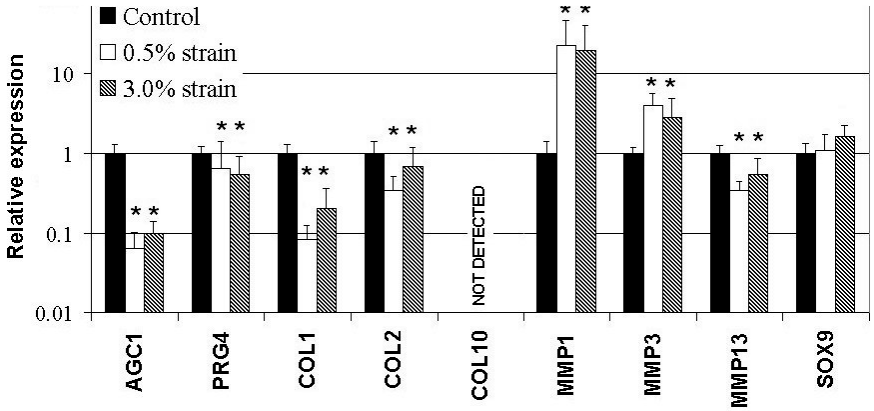

Figure 4: Gene expression of expanded, dedifferentiated chondrocytes under strain (0.5% and 3.0%) relative to unstrained controls. * indicates statistically significant difference with control (P<0.05).

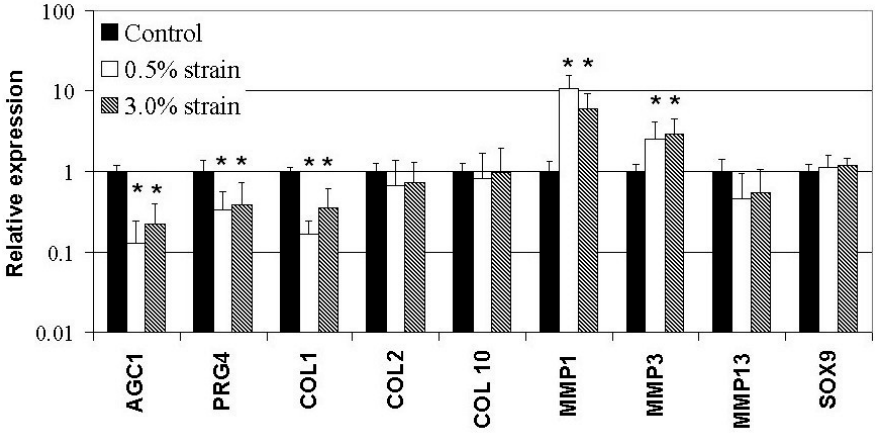

Figure 5: Gene expression of expanded, dedifferentiated chondrocytes in a chondrogenic environment under strain (0.5% and 3.0%) relative to unstrained controls. * indicates statistically significant difference with control (P<0.05)

stimulation of both 0.5% and 3.0% strain levels. Gene expression of AGC1 was only slightly altered, a 1.8-fold down-regulation was found at 0.5% strain and 1.6-fold change at 3.0% strain. COL1 showed a 3.8-fold

decrease in gene expression level compared to control at 0.5% strain and a 2.1-fold decrease at 3.0% strain. COL10 was also marginally down-regulated (2-fold) at both strain levels. Gene expression levels of COL2 remained unaltered when loaded with either 0.5% or 3.0% strain, while levels of PRG4 were slightly up-regulated compared to control, 1.8-fold at 0.5% and 1.9-fold at 3.0%. Stretch did neither statistically significantly alter the gene expression of MMP1, MMP3 and MMP13, nor did it change SOX9 expression levels.

Effect of stretch on expanded chondrocytes

P3 cells showed a much larger response to stretch (figure 4) than primary cells. Gene expression of most matrix proteins (AGC1, PRG4, COL1 and COL2) was severely down-regulated after stretching of the cells. AGC1 and COL1 showed the most significant change in gene expression. Expression levels of AGC1 were 15.6-fold lower at 0.5% when compared to unstretched controls, while 3.0% resulted in a 11-fold decrease. COL1 was down-regulated approximately 10-fold for both 0.5% (11.9-fold) and 3.0% strains (8.0-fold). Gene expression of PRG4 was also lower when cells were stretched at 0.5% (5.0-fold decrease) or 3.0% (2.5-fold decrease). After expansion, COL2 expression was absent in chondrocytes from one donor. In those cases where COL2 was still expressed, stretching downregulated its expression levels (up to 2.9-fold for 0.5% stretch). COL10 was not expressed in any donor after expansion. In expanded chondrocytes, MMP1 and MMP3 were both up-regulated after stretching. MMP1 showed a 20-fold upregulation while MMP3 was up-regulated 3.5-fold (at 0.5% strain) or 2.3-fold (at 3.0% strain). MMP13 was down-regulated in response to cell straining at both 0.5% (2.8-fold) and 3.0% (1.9-fold). Again, no effect of stretch on SOX9 gene was found.

Effect of stretch on expanded cells in a chondrogenic environment

On redifferentiation medium, expanded chondrocytes reexpressed COL2 and COL10, indicating a return to a more chondrogenic phenotype. COL1 and PRG4 expression was also higher on redifferentiation medium. Gene expression of MMP1 and MMP13 was also up-regulated on redifferentiation medium. This chondrogenic environment did not significantly change the alterations in gene expression levels of matrix components by expanded

Table 3
Summary of results

Gene	Condition and trend of relative expression			
	No stretch, P3[1]	Stretch, P0[2]	Stretch, P3[3]	Stretch, P3, redifferentiation medium[4]
AGC[1]	=	↓	↓↓	↓
PRG[4]	=	↑	↓	↓
COL[1]	↑	↓	↓↓	↓
COL[2]	↓↓↓	=	↓	=
COL[10]	ND	↓	ND	=
MMP[1]	↓	=	↑↑	↑
MMP[3]	↓↓↓	=	↑	↑
MMP1[3]	↓↓	=	↓	=
SOX[9]	↓↓	=	=	=

1Basal gene expression in expanded chondrocytes relative to primary controls (no strain, Fig. 2) and strain-related changes in expression in primary2, expanded chondrocytes3 and expanded chondrocytes stretched on redifferentiation medium4. Relative trends are indicated by symbols (Y, down-regulation; [, up-regulation; ¼, unchanged) with multiple arrows indicating stronger effects.
Single arrow: 0e10-fold change, double arrow: 10e100-fold change, triple arrow: 100e1000-fold change. ND, not detected.

chondrocytes associated with stretch (figure 5). AGC1 and COL1 were still severely downregulated, while PRG4 was again only moderately downregulated. COL2 was re-expressed on redifferentiation medium, but here stretch also appeared to down-regulate gene expression. No effect of stretch was found on mRNA levels of COL10 gene expression. MMP1 expression was still up-regulated, but to a lesser extent compared to basal medium. MMP3 expression was still significantly upregulated in stretched conditions compared to unstretched controls on redifferentiation medium. MMP13 was no longer significantly down-regulated. Overall an expression pattern was found that was similar to that found with basal expansion medium, but the effects seemed somewhat tempered. General trends for all conditions are summarized in Table 3.

Discussion

Our results indicate that in vitro expansion affects the response of chondrocytes to a mechanical stretch protocol. Real-time RT-PCR analysis revealed a decrease in expression of genes encoding for matrix components as well as a rise in expression of matrix degrading enzymes after stretching of expanded chondrocytes. In primary chondrocytes the response was markedly less substantial and significant. We also studied the effect of a chondrogenic environment that is known to direct dedifferentiated chondrocytes back toward a chondrogenic phenotype. These partially redifferentiated chondrocytes showed similar effects as the expanded, dedifferentiated chondrocytes, however, the effects of stretch appeared to be tempered. This is consistent with the shift toward the primary phenotype, since primary chondrocytes reacted only marginally to stretch.

The observation of up-regulation of the matrix degrading enzymes MMP1 and MMP3 after stretch is consistent with the notion that chondrocytes assume a more fibroblast-like phenotype upon dedifferentiation in respect that some studies report increased (pro-)MMP expression following (injurious) loading. For example, increased (pro-)MMP expression and activation was found in ligament fibroblasts [26], patellar tendon fibroblasts [27], scleral fibroblasts [28], uterine cervical fibroblasts [29] and cardiac fibroblasts [30] after loading with stretch. However, this up-regulation was not found in all types of fibroblast. Sambajon et al.[31] found no difference in proteinase activity of synovial fibroblasts after stretch. However, matrix degradation is also part of the remodeling process and it cannot be excluded that the rise in expression of MMP1 and MMP3 after short-term stretch follows from a remodeling attempt by the cells. But one might expect a concurrent elevation in matrix components in case of remodeling, which is not seen in our experiments. Obviously, the translation of changes in gene expression to expression on protein level is not straightforward and short term effects might differ from long-term (in vivo) effects. Therefore, to be able to interpret our results from a practical viewpoint, protein expression and enzymatic activity should be assessed and the consequences for long-term protein expression need to be established.

Interestingly, expression levels of MMP13, the collagenase whose affinity for COL2 is the greatest [32], were down-regulated after cyclic stretch. This difference in response might be attributed to the fact that the collagenases MMP1 and MMP13 differ in their spatial distribution [33]. MMP1 is mainly expressed in the superficial cartilage layer, while MMP13 is chiefly expressed in the deep zone, where different deformation is experienced by the chondrocytes. Other than very marginal changes in the expression of AGC1 and COL1 and COL10, primary chondrocytes did not show a marked response to stretch. This is in line with the findings of Millward-Sadler et al.[17], who found that expression levels of AGC1, MMP1 and MMP3 were unchanged in primary chondrocytes from OA patients after application of short-term cyclic stretch. The discrepancy with primary chondrocytes from healthy cartilage, which showed an increase in AGC1 expression and a decrease in MMP3 expression, was attributed to phenotypical alterations in OA chondrocytes. In OA chondrocytes, these changes might include altered expression of integrins, cytokines and growth factors. Indeed, integrins, and especially the fibronectin receptor integrin a5b1, are involved in mechanotransduction [34] of both normal and OA chondrocytes. Although the exact mechanisms by which this transduction occurs are not yet fully understood, they appear to include initiation of integrin-dependent signaling cascades. Expression of integrins and integrin-associated proteins is altered in chondrocytes upon expansion [35], which could account for the differences in response between primary and expanded chondrocytes found in this study.

Our model system utilizes monolayer culture with stretching (elongation) in the lateral direction, whereas the three dimensional in situ loading involves compression of the cells embedded in a matrix that includes solid, water and charges that control deformation upon loading. Knight et al.[36] showed that compression (of alginate) leads to contralateral elongation, although the exact deformation of the cell depends on the mechanical properties of the cell relative to its surrounding [37]. Plumb and Aspden [21] found that, contrary to healthy bovine cartilage, cyclic compressive loading was not stimulatory in cartilage biopsies from human femoral heads. Lee et al.[38] found decreased mRNA levels of COL2 and AGC1 following shear stress

loading of chondrocytes of patients suffering from OA. Also, Wiseman et al.[22] showed that, after three to four passages, healthy bovine articular chondrocytes seeded in agarose showed reduced glucosaminoglycan (GAG) synthesis after dynamic compressive loading. Nugent et al.[12] showed, with a shear deformation model, an up-regulation of PRG4 with the same order of magnitude as the primary chondrocytes in our stretch model system. Despite these consistent responses, the model systems never accurately represent the in vivo situation, where conditions such as deformation and environmental parameters are actively controlled. This limits the interpretation for the in vivo situation, where other factors, including cell attachment, molecular environment with different serum conditions and a complex loading situation might all influence the cell's response.

In conclusion, this study clearly shows that expanded human chondrocytes respond differently to stretch than primary chondrocytes. The down-regulation of both major components in articular cartilage, AGC1 and COL2, as well as the up-regulation of matrix degradative MMPs in the expanded chondrocytes after stretch might be regarded as degradative, although the effects of this altered gene expression on protein level still remain to be studied. If expanded chondrocytes in ACI-like procedures have a similar expression response after in situ loading, this cell source might not be the optimal choice for such a procedure. Consequently, it might be that other cell sources, redifferentiation protocols prior to implantation or limiting deformation (e.g., by movement restricting post-surgical therapy or use of a rigid scaffold) improve the cell's capacity to form a functional extracellular matrix and reduce enzymatic activity. However, implanted chondrocytes should also become involved in remodeling of the matrix, starting with degradation that might lead to better incorporation with the host matrix. From the current study, providing short-term RNA-level responses, we have no information regarding the long-term consequences for the matrix and its in situ incorporation potential. Our findings may therefore be regarded as a reference point for future studies that aim to optimize protocols for tissue formation by expanded chondrocytes.

References

1. von der Mark, K., et al., *Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture*. Nature, 1977. 267(5611): p. 531-2.
2. Mandl, E.W., et al., *Multiplication of human chondrocytes with low seeding densities accelerates cell yield without losing redifferentiation capacity.* Tissue Eng, 2004. 10(1-2): p. 109-18.
3. Schnabel, M., et al., *Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture*. Osteoarthritis Cartilage, 2002. 10(1): p. 62-70.
4. Guilak, F., A. Ratcliffe, and V.C. Mow, *Chondrocyte deformation and local tissue strain in articular cartilage: a confocal microscopy study.* J Orthop Res, 1995. 13(3): p. 410-21.
5. Sah, R.L., et al., *Biosynthetic response of cartilage explants to dynamic compression*. J Orthop Res, 1989. 7(5): p. 619-36.
6. Larsson, T., R.M. Aspden, and D. Heinegard, *Effects of mechanical load on cartilage matrix biosynthesis in vitro*. Matrix, 1991. 11(6): p. 388-94.
7. Kim, Y.J., et al., *Mechanical regulation of cartilage biosynthetic behavior: physical stimuli*. Arch Biochem Biophys, 1994. 311(1): p. 1-12.
8. Buschmann, M.D., et al., *Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture.* J Cell Sci, 1995. 108 (Pt 4): p. 1497-508.
9. Quinn, T.M., et al., *Mechanical compression alters proteoglycan deposition and matrix deformation around individual cells in cartilage explants*. J Cell Sci, 1998. 111 (Pt 5): p. 573-83.
10. Lee, D.A., et al., *The influence of mechanical loading on isolated chondrocytes seeded in agarose constructs.* Biorheology, 2000. 37(1-2): p. 149-61.
11. Bonassar, L.J., et al., *The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I.* J Orthop Res, 2001. 19(1): p. 11-7.
12. Nugent, G.E., et al., *Dynamic shear stimulation of bovine cartilage biosynthesis of proteoglycan 4*. Arthritis Rheum, 2006. 54(6): p. 1888-96.
13. Smith, R.L., et al., *Effects of fluid-induced shear on articular chondrocyte morphology and metabolism in vitro.* J Orthop Res, 1995. 13(6): p. 824-31.

14. Edlich, M., et al., *Oscillating fluid flow regulates cytosolic calcium concentration in bovine articular chondrocytes.* J Biomech, 2001. 34(1): p. 59-65.
15. Holmvall, K., et al., *Chondrocyte and chondrosarcoma cell integrins with affinity for collagen type II and their response to mechanical stress.* Exp Cell Res, 1995. 221(2): p. 496-503.
16. Akagi, M., et al., *Cyclic tensile stretch load and oxidized low density lipoprotein synergistically induce lectin-like oxidized ldl receptor-1 in cultured bovine chondrocytes, resulting in decreased cell viability and proteoglycan synthesis.* J Orthop Res, 2006. 24(8): p. 1782-90.
17. Millward-Sadler, S.J., et al., *Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes.* Arthritis Rheum, 2000. 43(9): p. 2091-9.
18. Salter, D.M., et al., *Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation.* Biorheology, 2002. 39(1-2): p. 97-108.
19. Salter, D.M., M.O. Wright, and S.J. Millward-Sadler, *NMDA receptor expression and roles in human articular chondrocyte mechanotransduction.* Biorheology, 2004. 41(3-4): p. 273-81.
20. Millward-Sadler, S.J., et al., *ATP in the mechanotransduction pathway of normal human chondrocytes. Biorheology,* 2004. 41(3-4): p. 567-75.
21. Plumb, M.S. and R.M. Aspden, *The response of elderly human articular cartilage to mechanical stimuli in vitro.* Osteoarthritis Cartilage, 2005. 13(12): p. 1084-91.
22. Wiseman, M., et al., *Passage in monolayer influences the response of chondrocytes to dynamic compression.* Biorheology, 2004. 41(3-4): p. 283-98.
23. Weyts, F.A., et al., *Mechanical control of human osteoblast apoptosis and proliferation in relation to differentiation.* Calcif Tissue Int, 2003. 72(4): p. 505-12.
24. Uitterlinden, E.J., et al., *Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants.* Osteoarthritis Cartilage, 2006. 14(3): p. 250-7.
25. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.*

Methods, 2001. 25(4): p. 402-8.
26. Zhou, D., et al., *Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts.* J Orthop Res, 2005. 23(4): p. 949-57.
27. Yang, G., H.J. Im, and J.H. Wang, *Repetitive mechanical stretching modulates IL-1beta induced COX-2, MMP-1 expression, and PGE2 production in human patellar tendon fibroblasts.* Gene, 2005. 363: p. 166-72.
28. Shelton, L. and J.S. Rada, *Effects of cyclic mechanical stretch on extracellular matrix synthesis by human scleral fibroblasts.* Exp Eye Res, 2007. 84(2): p. 314-22.
29. Yoshida, M., et al., *Prostaglandin F(2alpha), cytokines and cyclic mechanical stretch augment matrix metalloproteinase-1 secretion from cultured human uterine cervical fibroblast cells.* Mol Hum Reprod, 2002. 8(7): p. 681-7.
30. Tyagi, S.C., et al., *Stretch-induced membrane type matrix metalloproteinase and tissue plasminogen activator in cardiac fibroblast cells.* J Cell Physiol, 1998. 176(2): p. 374-82.
31. Sambajon, V.V., et al., *The effects of mechanical strain on synovial fibroblasts.* J Oral Maxillofac Surg, 2003. 61(6): p. 707-12.
32. Reboul, P., et al., T*he new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis.* J Clin Invest, 1996. 97(9): p. 2011-9.
33. Rannou, F., et al., *Cartilage breakdown in rheumatoid arthritis.* Joint Bone Spine, 2006. 73(1): p. 29-36.
34. Millward-Sadler, S.J. and D.M. Salter, *Integrin-dependent signal cascades in chondrocyte mechanotransduction.* Ann Biomed Eng, 2004. 32(3): p. 435-46.
35. Goessler, U.R., et al., *In vitro analysis of integrin expression during chondrogenic differentiation of mesenchymal stem cells and chondrocytes upon dedifferentiation in cell culture.* Int J Mol Med, 2006. 17(2): p. 301-7.
36. Knight, M.M., et al., *Cell and nucleus deformation in compressed chondrocyte-alginate constructs: temporal changes and calculation of cell modulus.* Biochim Biophys Acta, 2002. 1570(1): p. 1-8.
37. Guilak, F. and V.C. Mow, *The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage.* J Biomech, 2000. 33(12): p. 1663-73.
38. Lee, M.S., et al., *Effects of shear stress on nitric oxide and matrix protein*

gene expression in human osteoarthritic chondrocytes in vitro. J Orthop Res, 2002. 20(3): p. 556-61.

3

Chapter 3

Effects of individual control of pH and hypoxia in chondrocyte culture

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Published as Das RHJ et al., Effects of Individual Control of pH and Hypoxia in Chondrocyte Culture, J Orthop Res (2009)

Summary

Effects of oxygen tension (pO2) and pH on gene and protein expression and metabolic activity of human chondrocytes were independently assessed. Chondrocytes were cultured under a range of pH (6.4–7.4) and different pO2 (5 and 20%) during 5 days in a bioreactor. Effects on gene expression, DNA content, protein expression, and metabolic activity were determined.

Linear regression analysis showed that gene expression of type I collagen (COL1), SOX9, and VEGF is significantly lower at acidic pH, while expression of aggrecan, type II collagen, and HIF1A is pH-independent. Higher protein levels of VEGF were found under low pO2. Acidic pH severely lowered VEGF release into medium, glucose consumption, and lactate production. Extracellular pH proved to more potently influence cell function than oxygen tension, the latter showing down-regulation of COL1 gene expression and up-regulation of VEGF protein under hypoxia. Hypoxic culture inhibits COL1 mRNA expression pH-dependently, while expression of SOX9 is largely hypoxia independent, but pH dependent. Expression of HIF1A and VEGF revealed divergent pH dependencies.

Subtle fluctuations in extracellular pH and oxygen tension clearly influence chondrocyte metabolism and marker expression. Sophisticated pH and oxygen control not only allows study of (patho)physiological changes, but also opens new venues in cartilage tissue engineering.

Introduction

Compared to other cells, chondrocytes experience an acidic and hypoxic environment in vivo.[1] Because chondrocytes derive oxygen and nutrients from the surrounding synovial fluid (and to some extent from the underlying subchondral bone), oxygen tension in native cartilage is lower than in most other tissues. Estimates put the oxygen tension in cartilage at values around 7–10% in the superficial zone [2-4] and as low as 1% in the deeper zones,[1, 5] although there is also evidence that this fall is not as drastic in healthy cartilage.[6] In pathological conditions these values can drop even further, possibly due to elevated metabolic activity of the inflamed synovial tissue.[7] Under these anaerobic conditions, ATP production is limited to the energy equivalents that are generated by conversion of pyruvate to lactate. This acidic end product, in turn, lowers the pH of the extracellular environment. In degenerated spinal discs, for example, values below 6.5 have been measured.[8] Chondrocytes primarily utilize an anaerobic metabolism, even when enough oxygen is available.[9] Additionally, water expulsion during joint loading increases the ionic concentration, further reducing extracellular pH.[10]

Common tissue engineering approaches rely on mimicking the in vivo situation. Mimicking this hypoxic and acidic state in vitro does not automatically translate to improved tissue engineering results. In fact, the effect of hypoxia on proliferation, (re)differentiation, and matrix production is still a topic with much controversy. Low oxygen tension has beneficial effects on dedifferentiated bovine chondrocytes in alginate,[11] but effects appear to differ between species, differentiation state, and metabolic condition.[12] While the pH in cartilage in vivo is slightly acidic, a pH of approximately 7.2 seems to favor chondrocyte matrix synthesis.[13, 14] However, the mechanism by which pH influences matrix synthesis remains largely unknown. For instance, pH might alter growth factor function, cytokine expression, or limit incorporation of extracellular matrix components.[15, 16]
More than being just indicators for the state of a tissue culture, oxygen tension andpHthemselves can influence cell behavior. In bioreactor design, this moves the use of oxygen tension and pH determination from a purely

descriptive readout parameter to a tool to control the cell's metabolism. Knowing how to manipulate a cell's metabolism using environmental parameters that can be controlled in a bioreactor is invaluable in tissue engineering processes.

Successful cartilage tissue engineering relies on the proper balance between synthesis of its two prime matrix constituents (i.e., aggrecan and collagen type II) and their breakdown, which allows for remodeling. Aggrecan and collagen type II, whose transcription is in part controlled by transcription factor SOX9, make up the bulk of the extracellular matrix in healthy cartilage, while collagen type I is associated with osteoarthritic changes. Hypoxia inducible factor-1a (HIF1A) is a key transcription factor in chondrocyte survival under hypoxic conditions even in healthy chondrocytes.[17] However, one of its best known target genes, vascular endothelial growth factor (VEGF), might be involved in the pathophysiology of osteoarthritis (OA).[18] Its expression is linked to up-regulation of matrix-degrading matrix metallo-proteases (MMPs) in osteoarthritic chondrocytes[19] and bovine cartilage explants.[20]

Here, we introduce a bioreactor that allows independent control of extracellular pH and oxygen tension (pO2). Hence, effects of both parameters can be assessed accurately and independently in a semi high-throughputmanner. To understand how these parameters can influence chondrocyte function, we examined effects of pH and pO2 on gene and protein expression and secretable factors. These findings will aid in determining optimal conditions for bioreactor culture, as well as in understanding pathological processes in chondrocytes.

Materials and Methods

Micro-24 Bioreactor

For experiments into the effects of environmental parameters, a bioreactor system (Applikon B.V., Schiedam, The Netherlands) was tested that allows control over pO2 and pH (figure 1A). Because both pO2 and pH can be set, maintained, or adjusted for every individual well of a custom-made 24-well plate (cassette), multiple conditions can be tested in a single

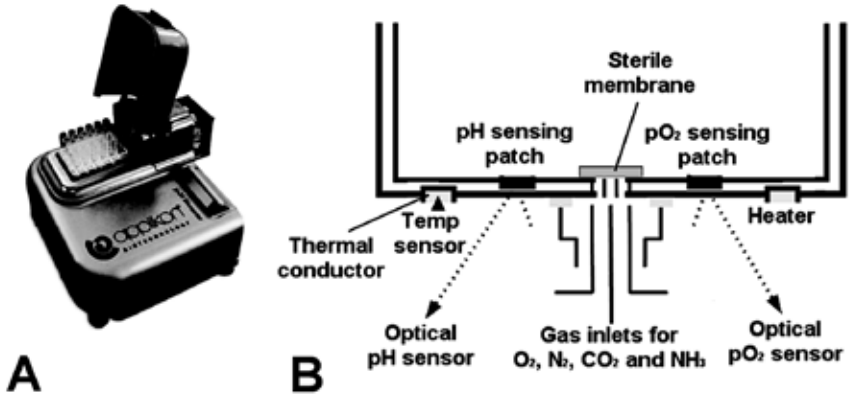

Figure 1: A: The micro-bioreactor. B: The complete lay-out of the sensory and control elements of the custom 24-well cassettes.

experiment.

pO2 and pH are measured through two fluorescent sensor patches (Presens, Germany) at the bottom of every single well on the custom-made 24-well plates, which function independent of medium composition. Individual well temperature is controlled through the use of a heater and temperature sensor, situated on the bioreactor. A hydrophobic membrane inlet in the center of the well allows control of pO2 and/or pH. pO2 can be controlled through one-way or two-way gas-input, depending on the oxygen demands of the culture. Hypoxic conditions are created through infusion of nitrogen (N2), while air or pure oxygen is used as the counter-gas. The pO2 setpoint is maintained with a proportional-integral-derivative (PID) control, whose settings for optimal accuracy and speed depend on the culture system used. Similarly, pH is controlled by infusion of CO2 (to lower pH) orNH3 (to raise pH). A complete overview of the bottom of a well is given in Figure 1B.

An orbital shaker system, together with sparging of control gasses, ensures homogeneity of the medium through mixing. As a result, depending on the nature of the culture system, addition of an antifoam agent might be required.

Oxygen control: accuracy measurements

We established the time needed to reach setpoint and subsequent fluctuations around setpoint during a 5 day period. Four milliliters of DMEM-HG (Invitrogen, Scotland) with 20 ppm Antifoam A (Sigma-Aldrich) was used in every well and shaker speed was set to 100 rpm. The pO2 was logged by the bioreactor at a sample rate of one measurement per minute, during 5 days. Within 30 min the pO2 was within 3% of the preset concentration. Deviations were limited to <1% pO2 over a culture period of 5 days for the lowest setpoint and increased slightly to 2% for the highest pO2.

pH measurements: agreement with standard reading and sensor drift

Phosphate-buffered solution (PBS; Gibco) was added to every well of a 24-well cassette. Temperature in each well was set to 37oC and ambient temperature under the hood of the bioreactor was kept at 35oC. Shaker speed was set at 500 rpm and neither pH nor pO2 control was activated. The pH was measured by the bioreactor every minute and pH data were logged every 20 min for 2 weeks and an offline measurement was performed daily for every single well using a Metrohm 713 pH meter (Metrohm, Herisau, Switzerland). Agreement between pH determination by the bioreactor and offline measurement using a pH-probe was assessed by the Bland-Altman method comparison procedure[21] using GraphPad Prism software. A strong correlation was found between the two types of measurement (Pearson r=0.9503). Bland-Altman analysis revealed that there was no consistent bias (mean ΔpH is 0) and normal distribution of ΔpH was found (limits of agreement are -0.1 and +0.1). Difference between online and offline measurements (ΔpH) did not increase in time and the average difference remained zero, which indicates that the measurement with light-sensitive fluorescent sensor patches did not suffer from photobleaching for up to 14 days.

Cell culture

Cartilage was obtained from two patients (ages 65 and 62) undergoing total knee replacement surgery for osteoarthritis (after approval by the local ethics committee; MEC2004-322). Full thickness cartilage was harvested,

treated with 0.2% protease in physiological saline solution (Sigma-Aldrich, Zwijndrecht, Netherlands) for 90 min and subsequently digested overnight in basal medium (DMEM, 4.5 g/L glucose with 10% fetal calf serum [FCS], 50 mg/mL gentamicine, and 1.5 mg/mL fungizone [all Invitrogen, Scotland]) supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a hemocytometer. The primary chondrocytes were then seeded at a density of 7,500 cell/cm2 in T175 culture flasks for expansion culture. The cells plated for expansion were cultured for three passages in basal medium. Alginate beads were created with third passage chondrocytes in 1.2% low viscosity alginate (Keltone LV; Kelco, Surrey, UK) by dripping a 1.2% alginate suspension with 4 million cells per mL though a 2300 gauge needle into a CaCl2 solution. After 10 min the beads were washed twice with physiological saline and once with basal medium and transferred to a T175 culture flask with 150 mL basal medium. After 4 days of preculture, 24 beads were transferred to every well of a 24-well plate, designed for use with a micro-24 bioreactor.

Experimental lay-out

A range of pH (6.4–7.4 with intervals of 0.2) was applied over 18 of 24 wells and pO2 was maintained at 5% pO2. The remaining six wells were kept at 20% pO2 at a pH of either 6.8 or 7.2 (three wells for every condition). The pO2 and pH control were both sampled every minute and data were logged every 5 min. The beads were cultured in 4 mL of chondrogenic medium (DMEM high glucose, 1:100 ITS, 10 ng/mL TGF-b2, 10 ng/mL IGF-1, 25 mg/ml L-ascorbic acid, and 50 mg/mL gentamicin and 1.5 mg/mL fungizone) supplemented with 20 ppm Antifoam A (Sigma-Aldrich). Alginate beads and medium were harvested for analysis after 5 days of culture, without refreshing the medium. Chondrocytes from two patients were used and every condition was performed in triplicate per donor (total of n=6 per condition).

DNA content

Nine beads per well were digested (33 beads) overnight at 56oC in papain digestion buffer (200 mg/mL papain in 50 mM ethylene diamine tetraacetate [EDTA] and 5 mM L-cystein hydrochloride). The amount of

DNA in each papaindigested sample was determined using an ethidium bromide assay with calf thymus DNA (Sigma-Aldrich) as a standard.

RNA isolation and quantitative RT-PCR

Per well, 15 alginate beads were pooled and dissolved in 150 mL/bead 55mM sodium citric acid and spun down for 8 min at 1,600 rpm at 4oC. Cell pellets were resuspended in 1.8 mL RNABee (TEL-TEST Inc., Friendswood, TX). After addition of 0.2 mL chloroform, samples were spun down for 20 min at 13,000g. Total RNA was isolated from the supernatant using the Qiagen RNA Micro Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop ND1000; Isogen Life Science, IJsselstein, The Netherlands). Both cDNA synthesis and real-time quantitative PCR methods were described earlier,[22] except that an ABI7000 was used for cycling.

Gene expression analysis of aggrecan (AGC1), collagen type I (COL1), type II (COL2), and type X (COL10), SOX9, HIF1A, and VEGF was performed. RT-PCR primers for aggrecan, collagen type I, II, X, and SOX9 were previously described.[23] Primers for 18S rRNA, HIF1A, and VEGF are listed in Table 1. Primer specificity was ensured by BLASTn search and presence of a single PCR product in agarose gel electrophoresis (data not shown). All assays were verified to have similar amplification efficiency. Relative expression levels, normalized to 18S rRNA as the most stably expressed of three reference genes (data not shown), were calculated using the $2^{-\Delta\Delta Ct}$ method.[24] Linear regression analysis using GraphPad Prism software was used to determine the relation between gene expression and extracellular pH. Differences between 5% pO2 and 20% pO2 as well as the difference between pH 6.8 and 7.2 under 20% pO2 were assessed with an unpaired Student's t-test using GraphPad Prism software.

Immunoblotting and ELISA

Presence of HIF1A protein was determined through Western Blot analysis. Total protein was isolated by successive organic extraction and subsequent alcoholic precipitation from the phenolic interphase obtained during RNA isolation. Protein was suspended in 0.75% w/v SDS and quantified using

Table 1
Oligonucleotide primers for 18S, HIF1A and VEGF

Gene	Target accession No.	Type	Oligonucleotide sequences (5' - 3')
18S rRNA	NR_003286.1	F	CGGCTACCACATCCAAGGAA
		R	GCTGGAATTACCGCGGCT
		P	TGCTGGCACCAGACTTGCCCTC
HIF1A	NM_001530.2	F	GGCGCGAACGACAAGAAA
		R	GATTCTTTACTTCGGCCGAGATCTG
	NM_181054.1	-	-
VEGF	NM_001033756.1	F	CTTGCCTTGCTGCTCTACC
	NM_001025370.1	R	CACACAGGATGGCTTGAAG
	NM_001025369.1	-	-
	NM_001025368.1		
	NM_001025367.1		
	NM_003376.4		
	NM_001025366.1		

SYBRGreen I assay (F = forward, R = reverse), Taqman assay incl. P= FAM-labeled probe.

BCA Protein Assay Reagent (23228; Pierce). Fifteen micrograms of total protein per condition was separated by SDS-gel electrophoresis on a 10% gel (Precise Protein Gel; Pierce). Proteins were electro-transferred to PVDF membranes (Roti-PVDF; Carl Roth, D-76185 Karlsruhe, Germany) using a semi-dry Trans-Blot SD system (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in 1X tris-buffered saline (TBS) containing 0.1% Tween-20 and subsequently incubated overnight at 4oC with the primary antibody (anti-HIF1α, sc-10790, 1:200; Santa Cruz). After incubation with secondary goat antirabbit-HRP conjugate (P-0448, 1:1,500; DAKO), antigen-specific signals were detected chemiluminescently (SuperSignal West Pico Substrate; Pierce).

Medium was spun down and supernatant stored at 80oC until analysis. One hundred microliter of medium was used for VEGF ELISA according to the manufacturer's instructions (DVE00; R&D Systems, Abingdon, UK). Linear regression analysis using GraphPad Prism software was used to investigate the relation between extracellular pH and VEGF release. Again, differences between 5% pO2 and 20% pO2 as well as the difference between pH6.8 and 7.2 under 20% pO2 were assessed with an unpaired Student's t-test using GraphPad Prism software.

Metabolic activity

Glucose and lactate concentrations in culture medium were determined using commercially available kits according to the manufacturer's instructions (respectively, Sigma-Aldrich and Trinity Biotech plc, Wicklow, Ireland). Glucose consumption was calculated by subtracting the determined concentration from the initial concentration in the medium. Linear regression analysis using GraphPad Prism software was used to investigate the relation between extracellular pH and glucose consumption and lactate production. Again, differences between 5% pO2 and 20% pO2 as well as the difference between pH 6.8 and 7.2 under 20% pO2 were assessed with an unpaired Student's t-test using GraphPad Prism software.

Results

Gene expression

Expression of several genes showed a clear dependence on extracellular pH (figure 2). Most notably, COL1, SOX9, and VEGF showed a significant correlation with extracellular pH, with lower pH inhibiting their expression (Table 2), while the expression of AGC1, COL2, and HIF1A was pH independent. COL10 was undetectable by PCR.

Aggrecan gene expression was independent of pO2 at pH 6.8 and 7.2 as 5 and 20% showed similar levels. Under hypoxic conditions, there was no clear correlation of aggrecan expression with extracellular pH. Collagen type I expression showed a strong dependency on extracellular pH under hypoxic conditions. Interestingly, a strong peak at pH 7.4 was found. In normoxic conditions this dependency on extracellular pH was even more pronounced, judging from the difference between pH 6.8 and 7.2 at pO2 20%. Moreover, a hypoxic environment seemed to inhibit collagen type I expression at higher extracellular pH (7.2). Collagen type II expression was low in all samples, which is related to the use of dedifferentiated chondrocytes. Consequently, variations between biological duplicates were large compared to other genes and no dependency on extracellular pH or pO2 was found.

For SOX9, low extracellular pH again seemed to inhibit gene expression under both hypoxic and normoxic conditions. A hypoxic environment did not alter gene expression compared to a normoxic environment under any extracellular pH. Contrary to its target gene, VEGF, HIF1A expression did not depend on extracellular pH. Somewhat counter intuitively, expression of HIF1A was lower under hypoxic conditions. As a whole, VEGF expression appeared higher at hypoxic conditions. Most interestingly, its expression was dependent on extracellular pH under hypoxia, again with lower extracellular pH inhibiting the expression of this growth factor.

HIF1A and VEGF are well known hypoxia-regulated genes. Because HIF1A expression is known to be controlled at the post-transcriptional level, we also examined its expression on a protein level. Additionally, the

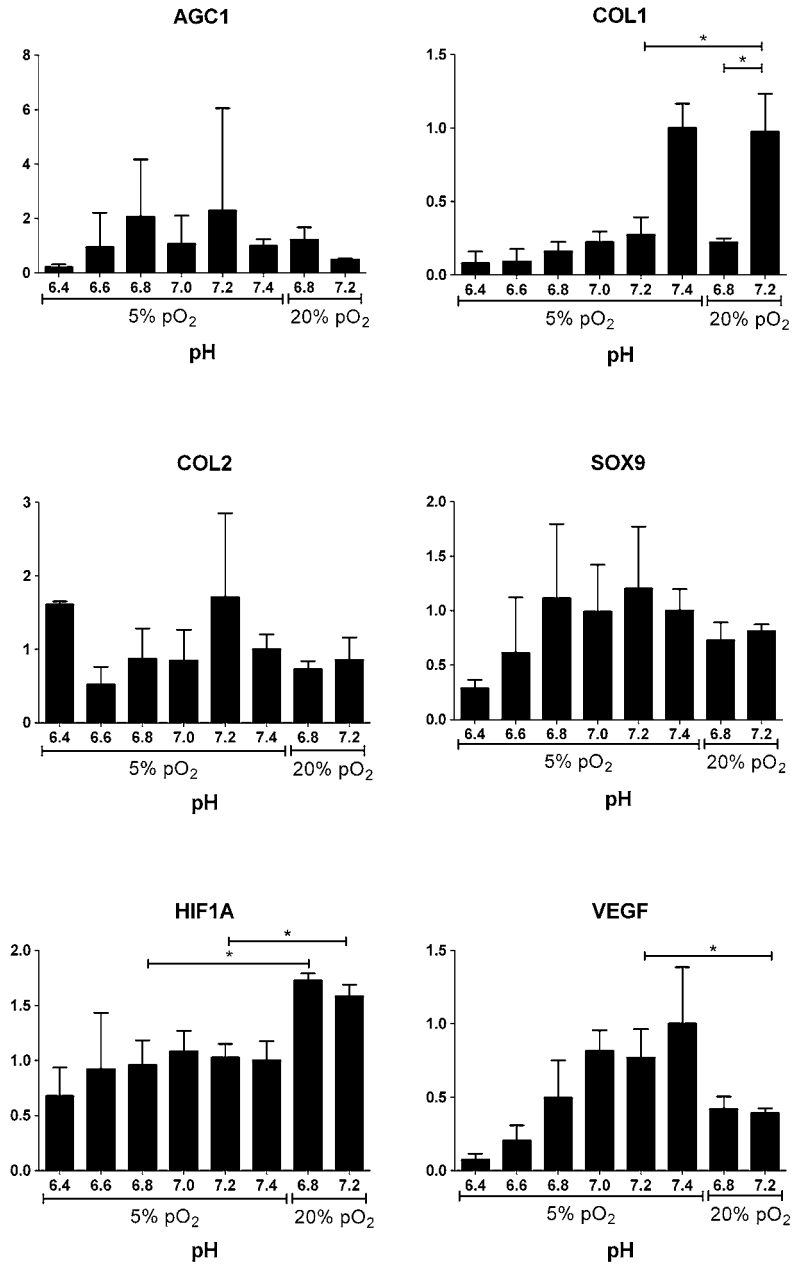

Figure 2: The pH-dependence of gene expression of chondrocytes in alginate beads: Relative mRNA levels for AGC1, COL1, COL2, SOX9, HIF1A and VEGF after five days of culture were determined by quantitative RT-PCR and normalized to 18S rRNA. .* indicates p-value <0.05 (N=6).

protein levels of VEGF were determined.

Protein expression

HIF1A Immunoblotting
HIF1A protein expression did not exhibit dependency on extracellular pH under either oxygen tension. After 5 days of culture, HIF1A was not significantly increased under 5% pO2 compared to 20% pO2 (data not shown).

VEGF ELISA
Following its gene expression, ELISA showed a strong dependency of VEGF release into the medium on extracellular pH under both oxygen tensions (figure 3). Moreover, lower oxygen tensions increased VEGF release compared to high oxygen levels with the same extracellular pH (figure 3). At extracellular pH 6.8, VEGF release was 50% higher at 5% pO2, compared to 20% pO2. At extracellular pH 7.2, VEGF release was 20% higher at lower pO2. At higher oxygen tension, the inhibitory effect of lower extracellular pH was already significant between pH 6.8 and 7.2.

Metabolic Activity
No significant difference in DNA content was found between the conditions in our experiments, indicating no excess cell death occurred during 5 day culture at low pH or pO2 (figure 4). In chondrocytes, both glucose consumption and lactate production depend on extracellular pH, but not on pO2 (figure 5A,B). Low pH inhibits glucose consumption and lactate production. Linear regression analysis showed a significant correlation between extracellular pH and glucose consumption and lactate production (Table 2). R2 was 0.45 and 0.87, respectively ($p<0.0001$).
The independence of oxygen is consistent with previous reports that chondrocyte metabolism is mainly anaerobic. Consequently, the expected mole ratio of lactate production over glucose consumption is ~2.0 (figure 5C).

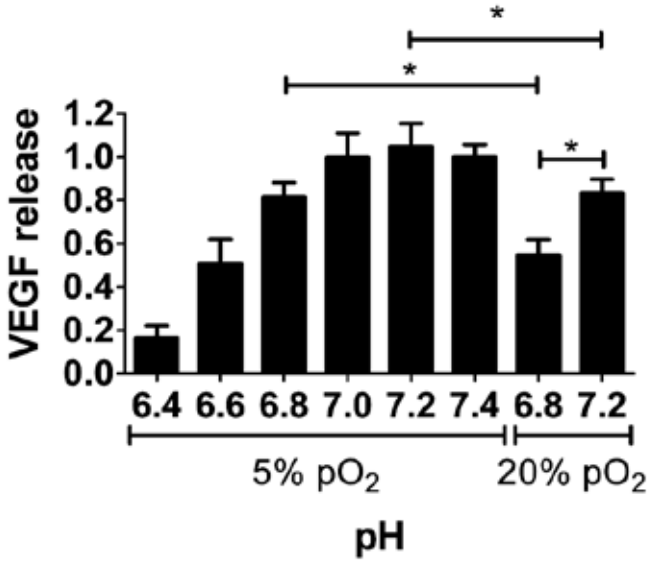

Figure 3: Relative VEGF release into the culture medium after five days at different pH levels and oxygen tensions, measured by ELISA.* indicates p-value <0.01 (N=6). Values are normalised to the average of pH 7.4 / pO2 5%.

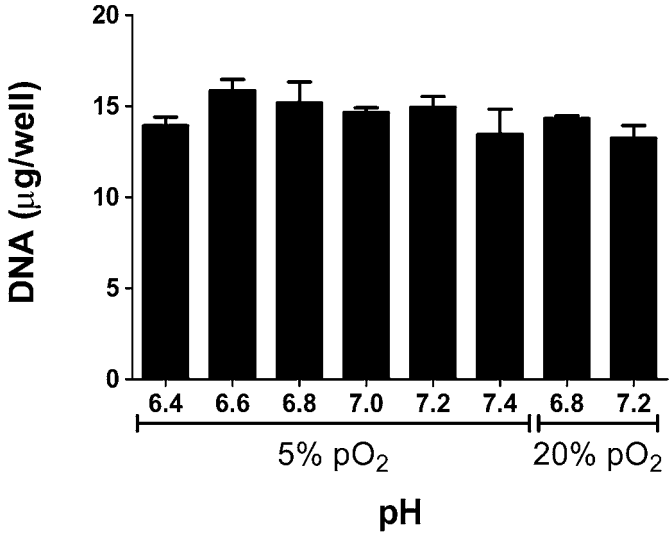

Figure 4: DNA content per culture well. DNA content (µg/well) is independent of extracellular pH or pO2, indicating that no differences in cell death or proliferation occurred under these conditions. (N=3).

Discussion

We introduced a novel bioreactor for studying environmental parameters and showed that chondrocyte culture is very sensitive to changes in environment. In our study, extracellular pH proved to more potently influence cell function than pO2. We showed that gene expression and metabolism can be severely inhibited by low pH. Similarly, growth factor synthesis (VEGF) was inhibited by a drop in extracellular pH, even within the physiological range. These results call for continuous monitoring and control of medium pH during in vitro culture for cartilage tissue engineering purposes.

Medium acidification during culture is common for most cell types and is usually counteracted with periodic refreshing. However, pH effects can be substantial even with small changes, which would warrant a change in frequency of refreshment or other means of extracellular pH control. In bovine chondrocytes, the effect of a range of pH was previously investigated by Wu et al.[14] In that study, inhibitions of low pH on lactate production and glycosaminoglycan (GAG) synthesis were observed in primary chondrocytes in agarose. The pH range investigated by Wu et al. (pH 6.6–7.3) had no effect on collagen synthesis or cell number. While our data confirmed this in that we could not find an effect on collagen type II transcription and proliferation (data not shown), we observed an altered expression of collagen type I. Because primary chondrocytes express collagen type II, rather than type I, this pH effect would not be apparent in primary cells. In contrast to primary chondrocytes, expanded chondrocytes shift their expression from primarily collagen type II to more collagen type I. Consequently, for passaged chondrocytes, pH-mediated effects on collagen expression might be more apparent for collagen type I. Effects on COL10 could not be assessed as the expression of this hypertrophic differentiation marker was undetectable in our cells.

Human chondrocytes have been shown to express acid-sensing ion channels as a means to sense changes in extracellular pH[25] and several pathways that govern intracellular pH recovery after exposure to acidic loads have been suggested for chondrocytes. A pivotal role has been

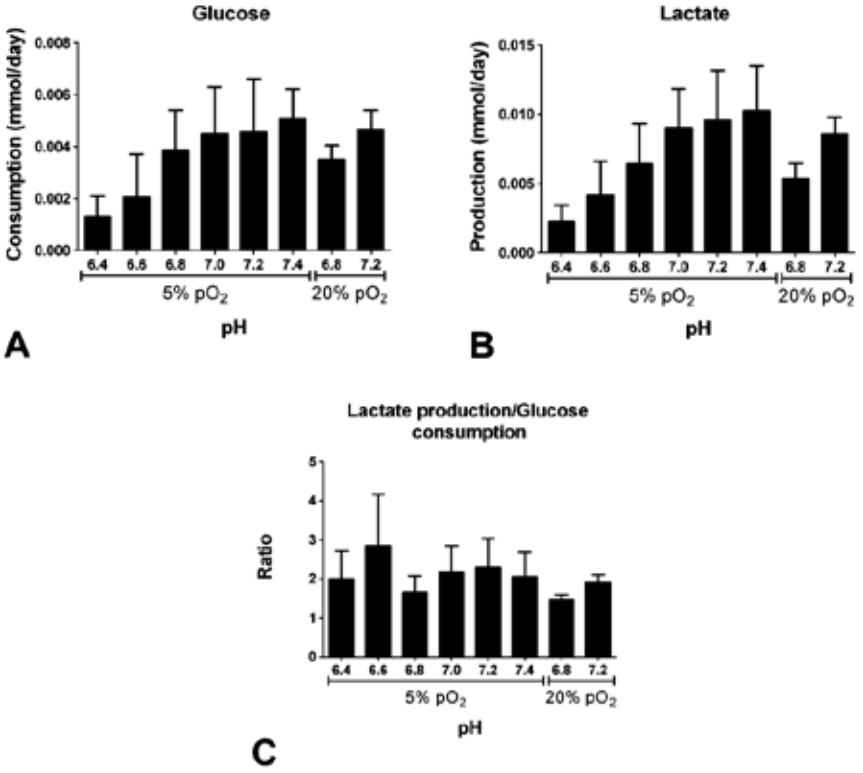

Figure 5: In cultured chondrocytes, glucose consumption (A) and lactate production (B) depend on pH, but not on oxygen tension. (C) The molar ratio of lactate production over glucose consumption. (N=6).

proposed for the Na+/H+ exchanger,[26, 27] but other systems like a Na+- and HCO3-dependent exchanger [28, 29] or the Na+-independent H+-ATPase exchanger[29] might also contribute. So far, it is unknown which of these mechanisms contributes the most to intracellular pH recovery in expanded chondrocytes.

Accurate control of extracellular pH and pO2 is valuable both in determining optimal conditions for regenerative medicine applications and investigating underlying biological mechanisms. In our study, pH was the more potent influence of the two environmental parameters studied. We tested 5% pO2 as it has been reported that this is the average pO2

Table 2
Overview of results

Parameter analysed	Linear regression coefficient (R^2)	p-value	Significant?
Aggrecan gene expression	0.03	0.30	No
Collagen type I gene expression	0.57	<0.0001	Yes
Collagen type II gene expression	0.03	0.35	No
SOX9 gene expression	0.16	0.022	Yes
HIF1A gene expression	0.08	0.12	No
VEGF gene expression	0.66	<0.0001	Yes
VEGF ELISA	0.78	< 0.0001	Yes
Glucose consumption	0.45	< 0.0001	Yes
Lactate production	0.87	< 0.0001	Yes

Linear regression analysis of pH effects assessed with GraphPad Prism® software. Linear regression coefficient R2 was determined for the pH range of 6.4-7.4 for gene expression, VEGF release, glucose consumption and lactate production. A p-value of <0.05 indicates a significant correlation with extracellular pH.

experienced by chondrocytes in vivo.[12] While control of pO2 is not a new concept, it is still a poorly understood aspect in chondrocyte metabolism and cartilage tissue engineering. Generally, one hypoxic condition is tested against the normoxic (20% pO2) and possibly anoxic condition. In cartilage tissue engineering, 5% pO2 is often used as the generic physiological, hypoxic tension experienced by chondrocytes in vivo. This distinction between a hyperphysiological condition (20% pO2) for chondrocytes and a pO2 that is used to represent the whole range of more physiologically relevant oxygenation levels might underestimate the biological effects this environmental parameter can exert. Previous findings by Lin et al.[30] indicated that HIF1A up-regulation in response to hypoxia in chondrocytes is significantly higher for pO2s that fall far below physiological levels for normal chondrocytes. Future research might focus on these effects in more detail. For this purpose, the bioreactor provides an excellent tool given its ability to accurately control pO2 over a wide range.

Low pO2 is associated with increased stability of HIF1α on protein level. HIF1α is degraded under normoxic conditions, but stabilized under hypoxic conditions, because the prolyl-hydroxylase that primes HIF1α for degradation uses oxygen as a cofactor.[31] Stabilized HIF1α dimerizes with HIF1β, allowing it to translocate to the nucleus where it can bind to promotor regions of hypoxia-responsive genes. Western blot analysis showed increased HIF1α protein levels under hypoxic conditions only at later time points, but no significant difference was found for the protein levels under varying pH (data not shown). However, we confirmed that small amounts of HIF1α can also be found under normoxic conditions.[32] VEGF, a well-known HIF1α target gene, was not only up-regulated under hypoxic conditions, but its expression was also higher at higher pH. The dependency of VEGF expression on extracellular pH may have relevant consequences: VEGF promotes angiogenesis and vascular invasion in late stage OA[33] and a lower environmental pH under pathological conditions may modulate vascularization and the progression of the disease. However, one should note that the PCR assay we used did not discriminate between VEGF variants, but rather detected all currently known VEGF variants. Further experiments are needed to shed more light

into the ^{22} dependency of the multiple VEGF isoforms.

Some of the effects of hypoxia on chondrocyte redifferentiation that were reported in earlier studies [5][34-37] might have been masked in this study by the use of growth factors that also induce redifferentiation. Hypoxia has been shown to increase TGF-b2 expression in other cell types, such as in human umbilical vein endothelial cells (HUVEC),[38] while in our study this growth factor was already added to the medium to induce redifferentiation.

In this article we introduced a novel bioreactor for the investigation of environmental parameters in cell and tissue culture. This bioreactor allows accurate and independent control of three important environmental parameters in cell culture, pO2, pH, and temperature. However, some technical issues, inherent in the bioreactor design, need to be addressed. Ammonia, which was used to maintain extracellular pH, is toxic in high concentrations (above 2 mM) at which it inhibits cell proliferation,[39] but significantly only at rather high pH values. Our experiments focused on sub-neutral pH values and ammonia concentrations were far below toxic levels and did not significantly influence proliferation (data not shown). Also, addition of small amounts of a chemical antifoaming agent is required to prevent foam formation caused by sparging of the control gasses. While high concentrations of antifoam can have an adverse effect on cell survival,[40] the amounts necessary were well below cytotoxic levels (data not shown).

We showed the effects pH and pO2 can (independently) exert on gene expression and metabolic level. The effects of pO2 and pH on cell cultures and their underlying mechanisms can be studied in a semi high throughput manner by applying a range of these parameters within one experiment. Our data show that modulating extracellular pH or pO2 even within the physiological range alters chondrocyte metabolism and the expression of relevant genes. Depending on the way gene expression and metabolic activity relate to cell function, pH and pO2 can be used to direct the culture process. Moreover, they might also give new insights into the underlying biological processes of endochondral ossification and the development

or progression of osteoarthritis. Understanding how cells react to signals imposed by their environment not only benefits tissue engineering efforts, where optimization of the culture process will lead to improved engineered constructs, it also aids our understanding of developmental and pathological processes.

Acknowledgements

This research was funded by the Dutch Program Tissue Engineering (DPTE, Project No. RGT.6738).

References

1. Silver, I.A., Measurement of pH and ionic composition of pericellular sites. Philos Trans R Soc Lond B Biol Sci, 1975. 271(912): p. 261-72.
2. Falchuk, K.H., E.J. Goetzl, and J.P. Kulka, Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. Am J Med, 1970. 49(2): p. 223-31.
3. Lund-Olesen, K., Oxygen tension in synovial fluids. Arthritis Rheum, 1970. 13(6): p. 769-76.
4. Treuhaft, P.S. and M.C. DJ, Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. Arthritis Rheum, 1971. 14(4): p. 475-84.
5. Grimshaw, M.J. and R.M. Mason, Bovine articular chondrocyte function in vitro depends upon oxygen tension. Osteoarthritis Cartilage, 2000. 8(5): p. 386-92.
6. Zhou, S., Z. Cui, and J.P. Urban, Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study. Arthritis Rheum, 2004. 50(12): p. 3915-24.
7. Dingle, J.T. and D.P. Thomas, In vitro studies on human synovial membrane; a metabolic comparison of normal and rheumatoid tissue. Br J Exp Pathol, 1956. 37(4): p. 318-23.
8. Kitano, T., et al., Biochemical changes associated with the symptomatic human intervertebral disk. Clin Orthop Relat Res, 1993(293): p. 372-7.
9. Lee, R.B. and J.P. Urban, Evidence for a negative Pasteur effect in articular cartilage. Biochem J, 1997. 321 (Pt 1): p. 95-102.
10. Mow, V.C., C.C. Wang, and C.T. Hung, The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. Osteoarthritis Cartilage, 1999. 7(1): p. 41-58.
11. Domm, C., et al., [Redifferentiation of dedifferentiated joint cartilage cells in alginate culture. Effect of intermittent hydrostatic pressure and low oxygen partial pressure]. Orthopade, 2000. 29(2): p. 91-9.
12. Malda, J., et al., Cartilage tissue engineering: controversy in the effect of oxygen. Crit Rev Biotechnol, 2003. 23(3): p. 175-94.
13. Wilkins, R.J. and A.C. Hall, Control of matrix synthesis in isolated

bovine chondrocytes by extracellular and intracellular pH. J Cell Physiol, 1995. 164(3): p. 474-81.
14. Wu, M.H., et al., Effect of extracellular ph on matrix synthesis by chondrocytes in 3D agarose gel. Biotechnol Prog, 2007. 23(2): p. 430-4.
15. Shi, Q., et al., Regulation of interleukin-8 expression by cellular pH in human pancreatic adenocarcinoma cells. J Interferon Cytokine Res, 2000. 20(11): p. 1023-8.
16. Xu, L. and I.J. Fidler, Acidic pH-induced elevation in interleukin 8 expression by human ovarian carcinoma cells. Cancer Res, 2000. 60(16): p. 4610-6.
17. Schipani, E., et al., Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev, 2001. 15(21): p. 2865-76.
18. Murata, M., K. Yudoh, and K. Masuko, The potential role of vascular endothelial growth factor (VEGF) in cartilage: how the angiogenic factor could be involved in the pathogenesis of osteoarthritis? Osteoarthritis Cartilage, 2008. 16(3): p. 279-86.
19. Enomoto, H., et al., Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. Am J Pathol, 2003. 162(1): p. 171-81.
20. Pufe, T., et al., Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. J Pathol, 2004. 202(3): p. 367-74.
21. Bland, J.M. and D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement. Lancet, 1986. 1(8476): p. 307-10.
22. Uitterlinden, E.J., et al., Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage, 2006. 14(3): p. 250-7.
23. Das, R.H., et al., In vitro expansion affects the response of chondrocytes to mechanical stimulation. Osteoarthritis Cartilage, 2008. 16(3): p. 385-91.
24. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001. 25(4): p. 402-8.
25. Jahr, H., et al., Identification of acid-sensing ion channels in bone. Biochem Biophys Res Commun, 2005. 337(1): p. 349-54.

26. Wilkins, R.J., J.A. Browning, and J.C. Ellory, Surviving in a matrix: membrane transport in articular chondrocytes. J Membr Biol, 2000. 177(2): p. 95-108.
27. Wilkins, R.J. and A.C. Hall, Measurement of intracellular pH in isolated bovine articular chondrocytes. Exp Physiol, 1992. 77(3): p. 521-4.
28. Browning, J.A. and R.J. Wilkins, Mechanisms contributing to intracellular pH homeostasis in an immortalised human chondrocyte cell line. Comp Biochem Physiol A Mol Integr Physiol, 2004. 137(2): p. 409-18.
29. Dascalu, A., Z. Nevo, and R. Korenstein, The control of intracellular pH in cultured avian chondrocytes. J Physiol, 1993. 461: p. 583-99.
30. Lin, C., et al., Hypoxia induces HIF-1alpha and VEGF expression in chondrosarcoma cells and chondrocytes. J Orthop Res, 2004. 22(6): p. 1175-81.
31. Jaakkola, P., et al., Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science, 2001. 292(5516): p. 468-72.
32. Grimmer, C., et al., Hypoxia-inducible factor 1alpha is involved in the prostaglandin metabolism of osteoarthritic cartilage through up-regulation of microsomal prostaglandin E synthase 1 in articular chondrocytes. Arthritis Rheum, 2007. 56(12): p. 4084-94.
33. Haywood, L., et al., Inflammation and angiogenesis in osteoarthritis. Arthritis Rheum, 2003. 48(8): p. 2173-7.
34. Domm, C., et al., Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage, 2002. 10(1): p. 13-22.
35. Grimshaw, M.J. and R.M. Mason, Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension. Osteoarthritis Cartilage, 2001. 9(4): p. 357-64.
36. Murphy, C.L. and A. Sambanis, Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes. Tissue Eng, 2001. 7(6): p. 791-803.
37. Murphy, C.L. and A. Sambanis, Effect of oxygen tension on chondrocyte extracellular matrix accumulation. Connect Tissue Res, 2001. 42(2): p. 87-96.
38. Akman, H.O., et al., Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. Blood, 2001.

98(12): p. 3324-31.
39. Schneider, M., I.W. Marison, and U. von Stockar, The importance of ammonia in mammalian cell culture. J Biotechnol, 1996. 46(3): p. 161-85.
40. Junker, B., Foam and its mitigation in fermentation systems. Biotechnol Prog, 2007. 23(4): p. 767-84.

4

Chapter 4

TGF-β2 is involved in the preservation of the chondrocyte phenotype under hypoxic conditions

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Manuscript submitted to Annals of Anatomy

Summary

Culturing chondrocytes in oxygen tension that more closely resembles their in vivo environment has been shown to have positive effects on matrix synthesis. In redifferentiation of expanded chondrocytes, hypoxia increased collagen type II immunostaining. However, the mechanism by which hypoxia enhances redifferentiation is still unknown. We employed novel bioreactor technology to investigate the role of TGF-β, a growth factor heavily implicated in matrix production, in chondrocytes under hypoxia.

Dedifferentiated chondrocytes in alginate were cultured for 48 hours under hypoxic (1% pO2) or normoxic (20%) conditions, using specialized bioreactor technology. Gene expression of hypoxia (GDF1-, PHD3, HAS2, VEGF, COX2) and chondrocyte markers (SOX9, COL2A1, COL1A1, AGC1 and MMP13), as well as components of the TGF-β signaling pathway (TGF-β isoforms, receptors, and downstream effectors) were analyzed by qPCR after 48 hours. In addition, protein expression of COL2 and TGF-β2 were evaluated. To further elucidate the involvement of the TGF-β2, we used siRNA and ALK5 inhibition.

Hypoxic culture showed robust upregulation of hypoxic markers as well as upregulation of SOX9 and COL2. TGF-β2 was the only isoform that was upregulated under hypoxia on both gene and protein level. In addition, both type I receptors (ALK1 and ALK5) were upregulated under hypoxia, but type II and III receptors were unresponsive. TGF-β2 siRNA abrogated the hypoxia-induced COL2 expression, as did ALK5 inhibition, giving a strong indication that this pathway is involved in chondrocyte redifferentiation under low oxygen tension.

Hypoxic culture is a common approach for cartilage tissue engineering, but its underlying mechanisms are still poorly understood. We show here that increased TGF-β2 signaling through ALK5 plays a role in hypoxia-induced redifferentiation of chondrocytes.

Introduction

Articular cartilage is covering the surface of all diarthrodial joints in the body where it provides frictionless articulation and resistance to compressive forces. Due to the avascularity of this tissue, chondrocytes rely on diffusion primarily from the surrounding synovial fluid for their supply of oxygen and nutrients. Consequently, articular chondrocytes reside in an environment that is naturally hypoxic compared to other tissues. A pO2 of 7-10% is estimated for the pericellular regions in the superficial zone of articular cartilage, while the pO2 might be as low as 1% in the deeper zones near the calcified cartilage [1-4]. Chondrocytes are well adapted for survival in this hypoxic environment. For example, they mainly use anaerobic metabolism for the generation of ATP, thereby minimizing consumption of oxygen [5]. However, chondrocyte isolation and subsequent expansion exposes these cells to oxygen tension that far exceed physiological values. Culturing chondrocytes under oxygen tensions that more closely resemble their in-vivo environment (i.e. “hypoxic”) has been shown to have positive effects on matrix synthesis. Increased proteoglycans (PG) and collagen synthesis has been found in several studies, although findings are not always conclusive. In fact, effects appear to depend on cell source, species and exact oxygen tension [6]. In healthy bovine chondrocytes, hypoxia showed increased mRNA expression of aggrecan (AGC1) and TIMP-1, but a slight decrease in collagen type II (COL2A1) mRNA at near anoxic condition in one study [7]. On the other hand, increased proteoglycan deposition and COL2 synthesis was found in another study [8]. During redifferentiation of expanded chondrocytes, low oxygen tension also appears to increase collagen type II immunostaining [9].

TGF-β appears to play a major role in cartilage repair and multiple micro-environmental factors are potentially regulating the chondrocytes phenotype directly or indirectly by modulating TGF-β signaling. TGF-β is the prototypic member of the TGF-β superfamily of growth factors, which further includes, among others, activins and inhibins, nodal, myostatin and bone morphogenetic proteins (BMPs) [10]. Three mammalian isoforms of TGF-β (1, 2 and 3) exist and while their active forms mostly have similar actions on cells in vitro, they also have non-redundant functions in vivo [11,

12]. This highly pleiotropic growth factor plays a key role in various cellular processes, including inhibition of cell growth, induction of apoptosis and production of extracellular matrix proteins.

In endothelial cells, hypoxia (1% pO2) was shown to upregulate TGF-β2 on the transcriptional level, peaking after 48 hours [13], while in dermal fibroblasts hypoxia upregulates the synthesis of the β1 isoform [14]. In redifferentiating bovine chondrocytes, complete anoxia appeared to slightly affect TGF-β expression [7], but this study did not distinguish between the different isoforms, nor did it investigate oxygen tensions between 0% and 5% pO2. Martin et al. [15] showed that in fresh bovine chondrocytes short term hypoxia (5% pO2) had a modulatory effect on TGF-β expression, but no longer-term effects were investigated, nor did the authors examine effects in human OA chondrocytes. . Other extracellular parameters also affect TGF-β signaling; while low pH can facilitate TGF-β signaling by activating its latent form [16, 17] other parameters, such as plasmin-mediated proteolysis [18], can also influence this signaling pathway [19]. The controversy around ambivalent effects of TGF-β on articular chondrocytes has been recently reviewed [20, 21] and appears to depend on the context of the culture: the presence of cytokines, the culture method (monolayer versus 3D culture) [22] or even the phase of the cell cycle [23]. In chondrocytes, TGF-β has been mainly implicated in the production of COL2 [24-26]. TGF-β2 has been described as the more potent stimulatory isoform (compared to TGF-β1) for chondrogenesis, proteoglycan synthesis and cell proliferation in rabbit chondrocytes [27]. TGF-β2 is also unique among the three isoforms in that it lacks a RGD integrin-binding sequence in its precursor [11] and is the only isoform with multiple CRE elements immediately upstream of its P1 promoter [11]. Of note, the CREB family of transcription factors has recently been shown to be activated by hypoxia [28].

Canonically, TGF-β ligands bind to the TGF-β type II receptor (TGFBRII) which, in most cells, then recruits the type I receptor activin-like kinase 5 (ALK5). The tetrameric receptor complex subsequently phosphorylates the intracellular effector molecules Smad-2 and -3, which, after translocation to the nucleus, regulate transcriptional activity in the target cell [29].

However, recently it was shown that in some cells (e.g. endothelial cells and chondrocytes) TGF-β can also signal through an alternative receptor, ALK1 [30]. Signaling via this receptor leads to phosphorylation of Smad1/5/8 rather than Smad2/3. It was postulated that the ratio of ALK5 and ALK1 signaling is responsible for the seemingly contradictory effects of TGF-β in chondrocytes [31]. In addition, Smad-independent pathways may fine-tune the response to TGF-β in different contexts [24]

In this study we hypothesize that hypoxia affects TGF-β signaling in redifferentiating human chondrocytes. Moreover, we postulate that the positive effects on COL2 expression in hypoxic culture of redifferentiating chondrocytes is caused by upregulation of components of the TGF-β signaling pathway. The different TGF-β isoforms as well as their receptors are investigated on gene expression and protein level, employing pharmacologic receptor inhibition and TGF-β2 RNAi to shed light on the underlying molecular effects.

Materials and Methods

Cell culture

Cartilage biopsies of 8 patients undergoing total knee replacement surgery (after approval by the local ethical committee; MEC2004-322) were used for this study. Full thickness cartilage was harvested, treated with 0.2% protease in physiological saline solution (Sigma-Aldrich, Zwijndrecht, Netherlands) for 90 minutes and subsequently digested overnight in basal medium (DMEM, 4.5 g/L glucose with 10% Fetal Calf Serum (FCS), 50 µg/ml gentamicine and 1.5 µg/ml fungizone (all Invitrogen, Scotland, UK)) supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a haemocytometer. The primary chondrocytes were then seeded at a density of 7,500 cell/cm2 in T175 culture flasks for expansion culture. The cells plated for expansion were cultured for three passages. Alginate beads were created with second passage chondrocytes in 1.2% low viscosity alginate (Keltone LV, Kelco, Surrey, UK) by dripping a 1.2% alginate suspension with 4 million cells per ml though a 23” gauge needle

into a CaCl2 solution. After 10 minutes the beads were washed twice with physiological saline and once with basal medium and transferred a T175 culture flask with 150 ml basal medium with 25 mM L-ascorbic acid. After five days of pre-culture beads were transferred to a µ24 bioreactor (Applikon Biotechnology, Schiedam, The Netherlands). Chondrocytes were also stimulated with 10 ng/ml TGF-β2 for 48 hours and 7 days under 1% or 20% pO2 and expression levels of key chondrocyte marker COL2 were compared to unstimulated controls.

Bioreactor culture

The bioreactor system used for hypoxic culture has been previously described by our group [32]. Briefly, 30 beads were transferred to every well of a specific 24 well cassette, designed for use with the µ24 bioreactor. Four milliliter of redifferentiation medium (DMEM, 4.5 g/L glucose with 1:100 ITS+ premix, 10 ng/ml IGF-1, 25 µg/ml L-ascorbic acid and 50 µg/ml gentamicin and 1.5 µg/ml fungizone) was added to every well. Oxygen tension was lowered by continuous sparging with nitrogen. Oxygen tension was maintained at the desired set-point (either 1%, 5% or 20% pO2) by using air as a counter gas. Medium pH was maintained at 7.2 using CO2.

RNA isolation and quantitative RT-PCR

Alginate beads were produced as described earlier by our group [33, 34] and after dissolution cells were resuspended in 350 µL RNABee (TEL-TEST Inc., Friendswood, TX, USA). After addition of 60 µl chloroform, samples were spun down for 20 minutes at 13,000xg. Total RNA was isolated from the supernatant using the Qiagen RNA Micro Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop® ND1000, Isogen Life Science, IJsselstein, The Netherlands). In compliance with MIQE guidelines [35], good RNA integrity (RIN ≥8.8; BioAnalyezr 2100, Agilent Technologies, Amstelveen, Netherlands) and absence of PCR inhibitors (data not shown) was ensured. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Thermo Fisher Scientific Inc., Rockford, IL) with mixed priming (oligo-d(T):random hexamers, 3:1 v/v) according to the supplier's instructions.

Both cDNA synthesis (0.5-1 g total RNA/reaction) and real-time quantitative PCR (10-15 ng cDNA/reaction) method, with specificity and efficiency controls, as well as data normalization to a reference gene index were described in detail elsewhere [36] . Briefly, the geometric mean of CT-values of pre-evaluated endogenous calibrators 18SrRNA, beta-2 microglobulin (B2M), ubiquitin (UBC), beta-actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and beta-2 microglobulin (B2M) [37] was used to normalize expression data. Relative expression was then calculated using the 2- Ct method [38] as described previously by us [36]. RT-PCR primers of ECM components (aggrecan, collagen type I and type II, SOX9, matrix metalloproteinase-13 (MMP13), markers of hypoxia (PHD3, VEGF, GDF10, HAS2 and COX2), TGF-β isoforms (TGF-β1, -β2 and -β3), TGF-β receptors (ALK1, ALK5, TGFBR2, BGCAN and EGN) and targets of TGF-β signaling (plasminogen activator inhibitor-1 (PAI-1) and inhibitor of DNA binding 1 (ID1)) were also previously described [36, 39-41]. Cycling was performed on an ABI7000 (Applied Biosystems) with 60°C annealing and a standard two-step protocol.

Immunoblotting

After RNA isolation, polypeptides were purified from the phenol-ethanol supernatant layer left over by dialysis. Briefly, upon 1:4 dilution, pooled phenol layers from three independent bioreactor experiments were dialyzed against 0.2% (w/v) SDS for 16, 4, and 2 hours, respectively, using Spectra/Por®3 (Carl Roth GmbH & Co.KG, Schoemperlenstr., Karlsruhe, Germany). Subsequently, dialysates were centrifuged (10,000 x g, 10min, 4°C) and supernatants concentrated using Centriprep YM-3 (Millipore, Billerica, MA) according to the manufacturer's instructions. Polypeptide concentration was determined using routine bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific Inc., Rockford, IL) as described earlier [42] and immunoblotting was performed as described elsewhere by our group [43]. Concisely, equal amounts of polypeptides were separated by routine SDS-PAGE, subsequently transferred by electro-blotting and detected on an Odyssey infrared imaging system (Li-Cor Biosciences) as described earlier [44], but using primary polyclonal goat anti-Col2a1, polyclonal rabbit anti-Sox9 [43] and mouse monoclonal anti-β-tubulin (G-8; Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies. For dot

blot analyses, the pooled polypeptide fractions were spotted onto 0.45 µm nitrocellulose membranes using a Bio-Dot® Apparatus (both: Bio-Rad, München, Germany) and processed as described above for Western blots, except that an anti-human TGF2-specific antibody (Proteintech, Chicago, IL; 1:1,000) was used. Densiometric quantification data (ImageJ software, version 1.47) were normalized to β-tubulin and displayed relative to controls, as reported by Caron et al. [43].

ALK5 inhibition

ALK5 activity was inhibited using a novel, specific ALK5 inhibitor (SB-525334, Sigma-Aldrich; 10 µM) [45] added two hours prior to induction of hypoxia (1% pO2). Ten beads were harvested after 48 hours of hypoxia for RNA isolation as described above. Specificity of the inhibitor was confirmed using qRT-PCR analysis on ALK5 and ALK1 specific target genes *PAI-1* [46, 47] and ID1 [31, 48] , respectively.

RNA interference (RNAi)

We used our established lentiviral vector technology for nontransient shRNA-mediated gene silencing in primary chondrocytes [36], employing commercially available, pre-validated shRNA clones (Sigma-Aldrich, MISSION shRNA library). Briefly, BamHI/MunI restriction fragments of the parental pLKO.1-puro vector containing one out of five different, sequence-verified anti-human TGF- 2 shDNAs were subcloned into corresponding restriction sites of recipient vector pRRL.PPT.PGK. GFPpre. Lentiviral particles were produced in HEK293T cells by transient transfection using a calcium phosphate protocol. Cells transduced with a lentiviral vector lacking the TGF 2-specific shRNA expression cassette (CTRL) and a non-targeting scrambled shRNA sequence (SCR), which did not significantly differ from CTRL (not shown) [36], served as controls. Quantitative RT-PCR was subsequently used to determine the knockdown efficacies: TRCN0000033425 (RNAi-1) and TRCN0000033427 (RNAi-2) were selected as best performing shDNA clones, reaching 75-80% of NM_003238 mean knock-down efficacy at a MOI of 1.5 in HEK29T cells (data not shown). In subsequent experiments, P1 OAHACs from three donors were seeded and transduced at a MOI of 2 with RNAi-1 and RNAi-2 for ± 18 hours as described earlier [36]. Cells were then used as passage

2 cells in monolayer culture (10,000 cells/cm2) and cultured under hypoxia or normoxia for 48 hours.

Results

Hypoxia and chondrogenic markers

To validate our culture system, we first confirmed the expression of accepted marker genes that have been shown to be hypoxia-responsive in different species and in vitro settings [40, 49-51]. We, therefore, used these genes as robust indicators of cellular hypoxia on mRNA level: a highly significant upregulation was found for four out of five of these markers under 1% pO2 (figure 1A). When compared to the 20% pO2 "normoxic" controls, PHD3 and VEGF were the most hypoxia responsive, displaying a more than 20-fold upregulat ion under 1% pO2, while GDF10 and COX2 were upregulated almost 7-fold. Expression of HAS2 was not significantly affected by hypoxia, although data suggested its responsiveness to hypoxia in chondrocytes [40, 52].

Next, we studied the expression levels of selected chondrocyte differentiation stage specific markers: hypoxia induced COL2A1-specific transcription factor SOX9 [53-55] significantly (4-fold), along with that of COL2A1 (6-fold) itself. In contrast, collagen type I (COL1A1) expression was downregulated under the same condition (figure 1B), leading to an improved COL2/COL1 ratio (data not shown). Interestingly, we could only detect a non-significant trend towards upregulation of the core protein of key proteoglycan (PG) aggrecan (AGC1) after 48 hours of low oxygen pressure. Of note, the key chondrocyte hypertrophy marker matrix metalloproteinase 13 (MMP13) [56] showed a non-significant trend towards downregulated under these conditions. We further verified the expression of key chondrocyte phenotype markers, SOX9 and COL2, on protein level by Western blot analysis and confirmed a significant, more than 3-fold, upregulation of both (figure 1C).

Hypoxia mediated TGF-β isoform expression

Since the TGF-β system plays a crucial role in cartilage homeostasis and repair, we investigated its oxygen pressure-dependent regulation in articular chondrocytes. Of all three human isoforms, hypoxia specifically

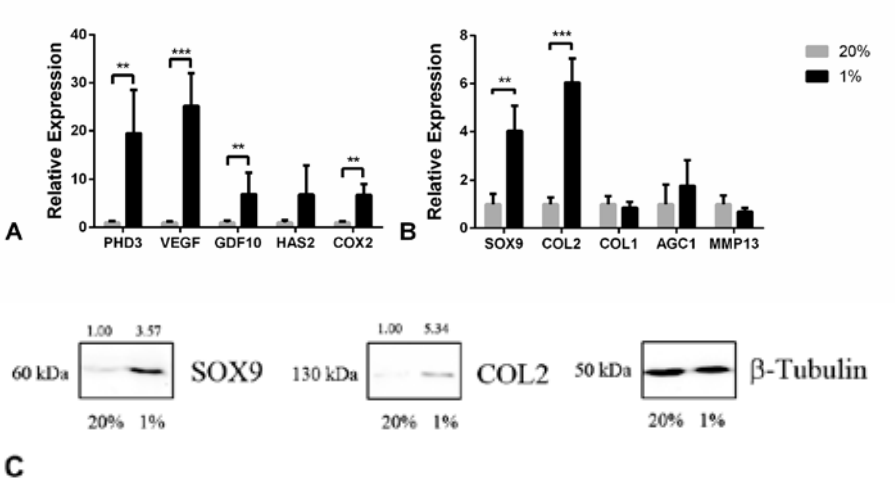

Figure 1: Relative gene expression of hypoxia- and chondrocyte-specific markers. Expression of accepted indicators of hypoxia in chondrocytes (A) next to that of chondrocyte differentiation-specific markers (B) after 48h in bioreactor culture. Representative protein expression of SOX9, collagen type II (COL2A1) and β-tubulin (loading control) (C). Hypoxia (1% pO2, black bars) and normoxia (20% pO2, grey bars). Molecular weight markers (kDa) are indicated on left and normalized quantitative data above respective bands. ** $p<0.01$, *** $p<0.001$.

only induced TGF-β2 expression in human articular chondrocytes: Following 48 hours of 1% pO2 TGF-β2 mRNA was significantly up-regulated by 60% as compared to the normoxic control (20% pO2). Expression of the other two isoforms (TGF-β1 and -β3) was not altered under these conditions (figure 2A). Subjecting human chondrocytes to 5% pO2 did also not significantly alter the expression of TGF-β isoforms (data not shown). Consequently, we further focused on comparing 1% pO2 to 20% pO2. To verify if the detected changes on mRNA level could be biologically meaningful, we also performed TGF-β2 immunoblots to demonstrate a similar magnitude of regulation on protein level (figure 2B). Exogenously applied TGF-β2 is known to induce COL2 expression in rabbit perichondrial cells cultured in alginate beads [57], but little is known about its effects under hypoxic conditions. Therefore, we tested

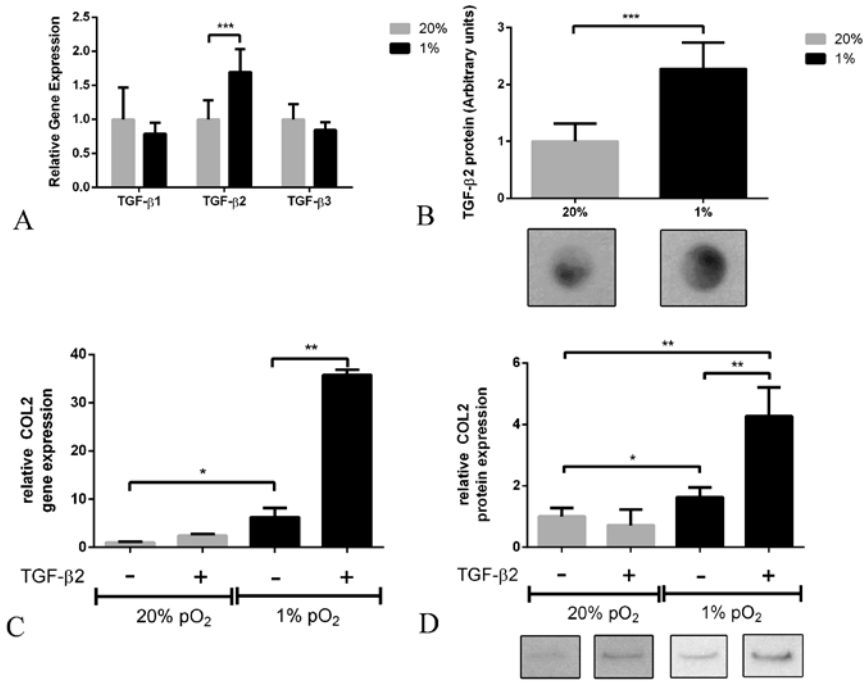

Figure 2: Oxygen tension-dependent changes in gene and protein expression of TGF-β and collagen. Relative mRNA abundances of the three mammalian TGF-β isoforms after 48 hours (A). Expression of TGF-β2 protein under normoxia and hypoxia with representative blots below (B). Effects of hypoxia and stimulation with exogenous TGF-β2 (10 ng/mL) on the induction of COL2A1 mRNA (C) and COL2 protein expression (D). Representative immunoblot signals are shown below their respective quantification chart. Hypoxia (1% pO2, black bars) and normoxia (20% pO2, grey bars).* $p<0.05$ ** $p<0.01$ *** $p< 0.001$.

if this isoform would be able to also induce COL2 expression in human articular chondrocytes. Under normoxic conditions (20% pO2) exogenous TGF-β2 did not significantly upregulate COL2 mRNA expression (figure 2C). In contrast, as shown before (figure 1B), hypoxia itself was able to induce COL2 expression to some extent. However, there was a prominent synergistic induction once exogenous TGF-β2 was applied under hypoxic conditions: COL2A1 mRNA was significantly induced 5.7-fold. On protein

level, a similar trend was confirmed, resulting in a synergistic 4-fold induction of collagen type II under low oxygen pressure in the presence of TGF-β2 (figure 2D).

Hypoxia and TGF-β receptor expression

To further shed light on the hypoxia-mediated TGF-β signaling, we looked into the expression of relevant TGF-β receptors. Hypoxic culture led to a significant upregulation of the expression of both type-1 receptors, ALK1 and ALK5 (figure 3A), which more than doubled (approximately 120% increase) compared to the normoxic condition. In contrast, expression of the type II (TGFBR2) and type III receptors (BGCAN and EGN) was unaffected by changes in oxygenation levels (figure 3B and 3C).

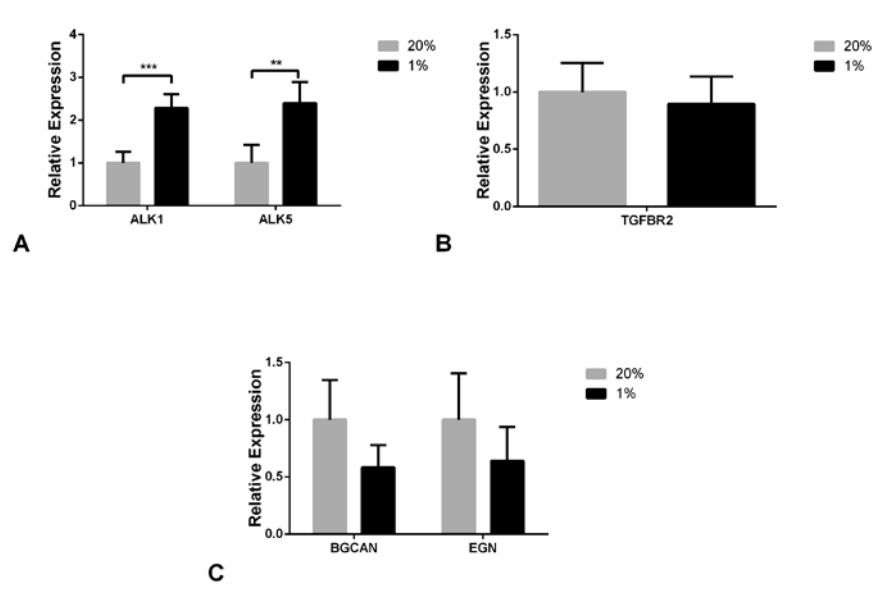

Figure 3: Oxygenation-dependent expression of TGF- β receptors. Type I receptors ALK1 and ALK5 (A) next to type II receptor (B) and III receptors (C). Hypoxic conditions (1% pO2) are shown as black bars and normoxic conditions (20% pO2) as grey bars. ** $p<0.01$, *** $p<0.001$

TGF-β signaling interference

Hypoxia induced COL2 expression in human articular chondrocytes in vitro and TGF-β2 was the only isoform of this cytokine that was also induced upon oxygen deprivation in these cells. In addition, applying exogenous TGF-β2 (10 ng/mL) to these cells synergistically induced collagen type II expression on mRNA and protein level (figure 2C/D) exclusively under hypoxic conditions. Therefore, we used isoform-specific shRNAs to investigate if a specific knockdown of TGF-β2 could selectively suppress COL2A1 levels under hypoxic conditions.

First, we evaluated the knockdown efficacy of five commercially available shRNA sequences by screening residual TGF-β2 expression levels in HEK293 cells as reported earlier [36]. In our hands only two constructs (RNAi-1 and -2) reached at least 70% knockdown efficacy (data not shown) and were chosen for further experiments using primary human articular chondrocytes. In primary chondrocytes we first verified the TGF-β isoform specificity and knockdown efficacy of the two most efficient shDNAs constructs 48 and 96 hours, respectively, after lentiviral transduction (figure 4A, only RNAi-1 is shown). RNAi selectively suppressed only TGF-β2 expression without negatively affecting the other two mammalian TGF-β isoforms. The shRNA expression was shown to result in a stable, about 70% (as compared to non-targeting controls, normalized to 1), efficient knockdown in cells 48 hours after transduction and 48 hours later (96h) in alginate beads.

In primary human chondrocytes in alginate beads in a bioreactor culture system, hypoxic conditions alone non-significantly ($p=0.0556$) upregulated COL2 mRNA expression in the non-targeting controls (CTRL). Addition of exogenous TGF-β2 (10 ng/ml) to these hypoxic cells prominently and synergistically increased COL2 expression even further (figure 4B). In contrast, effective TGF-β2 RNAi by means of non-transient lentiviral shRNA expression (RNAi-1 and -2) significantly suppressed this induction in COL2 expression. The TGF-β2 RNAi suppressed the COL2 expression to below that of the non-targeting control (figure 4B).

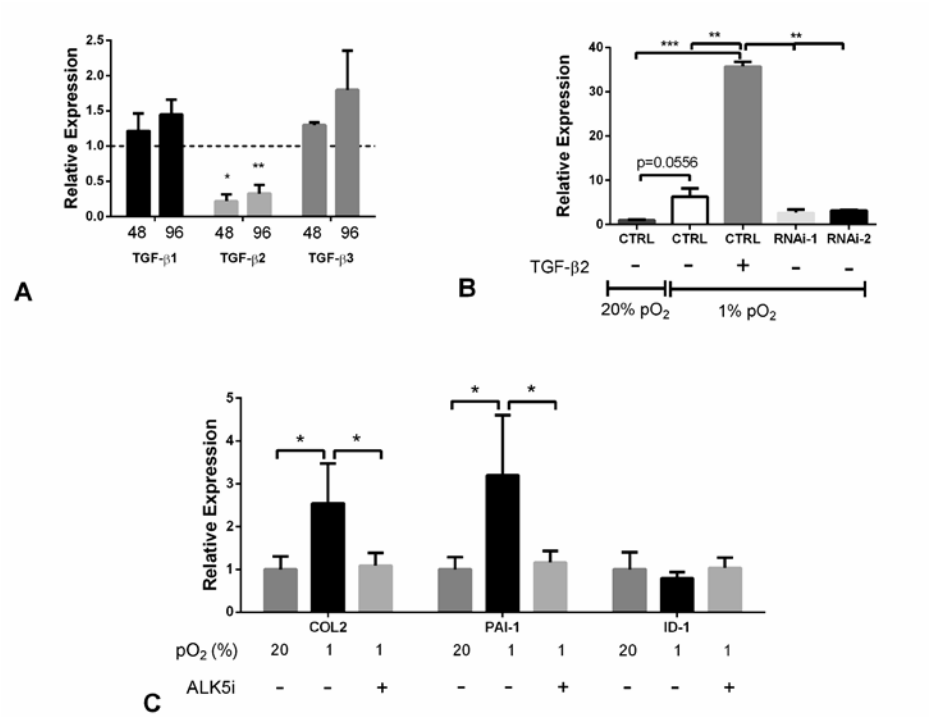

Figure 4: Specificity and efficacy of TGF- β 2 RNAi and TGF- receptor inhibition. Confirmation of the TGF-β isoform specificity and knockdown efficacy of the lentiviral shDNA vectors in primary chondrocytes (A). Suppression of hypoxia-induced and TGF-β mediated COL2 expression in primary chondrocytes by TGF-β2 RNAi (B). A specific small molecular receptor antagonist (SB-525334, ALK5i) was used to confirm ALK5 participation in the TGF-β2 mediated downstream signaling. PAI-1 and ID-1 served as established TGF-β inducible, but strictly ALK5- and ALK1-dependent, controls (C). Hypoxic conditions (1% pO2) are shown as black bars and normoxic conditions (20% pO2) as grey bars. * p<0.05, ** p<0.01, *** P<0.001

Finally, we aimed at identifying the receptor that mediates this TGF-β2-dependent upregulation of COL2 expression under hypoxic conditions. To this end, we used a highly ALK5-specific, novel type I receptor inhibitor under the same experimental conditions. While hypoxia, again, reproducibly induced COL2 expression under hypoxic conditions, the presence of the small molecular ALK5 antagonist SB-525334 (ALK5i)

fully abrogated this effect (figure 4C): COL2 induction was suppressed to control levels. Interestingly, in contrast to blocking TGF-β2 itself, COL2A1 mRNA levels were indistinguishable between normoxic controls and the hypoxic condition with ALK5i. The established TGF-β-inducible and ALK5-dependent expression of PAI-1 [30, 31] revealed a very similar pattern to that of COL2A1. The expression of ID-1, which is reported to be TGF-β-inducible but in chondrocytes ALK1-dependent [46], was not induced by hypoxia nor affected by ALK5i (figure 4C).

Discussion

In the present study, we used a sophisticated bioreactor system to stringently feedback-control the dissolved oxygen concentration in culture media of 3D-cultured chondrocytes to show that hypoxia (1% pO2) induces COL2A1 expression. We further hypothesized that hypoxia facilitates TGF-β signaling, which via ALK5, synergistically contributes to an improved phenotype by inducing the expression of key chondrocyte marker COL2A1 in redifferentiating human chondrocytes.

We first validated our culture system and confirmed the robust expression of well established hypoxia-responsive marker genes (figure 1A). Hypoxia inducible factor, HIF, expression and transcriptional activity are regulated by oxygen-sensitive prolyl hydroxylases (PHD1-3), of which PHD3 was shown to be most prominently regulated by sustained hypoxic culture in other cells [58]. We confirmed its strong, 20-fold, induction in hypoxic chondrocytes. Others reported VEGF, GDF10, HAS2 and COX-2 (PTGS2) as hypoxia responsive genes [40, 49-51]. Expression of vascular endothelial growth factor, VEGF, is hypoxia-inducible in cartilage cells [59], but the magnitude of its induction (20-fold, figure 1B) was unexpected. Lafont et al. [40] earlier reported a rather moderate, approximately 3-fold induction, of this gene, which may be an underestimation due to their microarray-based analysis or result from the fact that these authors analyzed healthy human chondrocytes from osteosarcomas. The fold-change of GDF10 and COX-2 expression in the present study is in nice agreement with earlier reported QPCR data [40].

Hyaluronan, a key component of the chondrocytes' ECM, is synthesized by a family of hyaluronic acid synthetases (HAS). HAS2, which is a particularly important isoform, was upregulated by hypoxia in our cultures, but just not significantly due a large standard deviation. Others [40, 52] also reported its positive hypoxia-dependency, although the fold change of hypoxia-induced HAS2 regulation was moderate (<3-fold). We further evaluated the hypoxia-dependency of selected other components of the chondrocytes' ECM, like SOX9, collagens, key proteoglycan aggrecan and hypertrophy marker MMP13 (figure 1B). COL1 expression was not-significantly regulated in our study, but slightly, about 50%, suppressed in another study using human chondrocytes in hypoxic 3D-culture [52]. The trend towards upregulation of AGC1 under 1% pO2 in our study was not significant. This may be a timing issue as AGC1 expression seems to respond early [40]. Overall, AGC1 seems to be only moderately induced by hypoxia [40, 52], which is apparently sufficient to double the sGAG content in cultured human chondrocyte pellets [52] and the trend in our data may thus still be biological meaningful. The magnitude of hypoxia-induced fold changes in SOX9 and COL2A1 expression in human chondrocytes seem to be rather robust and similar between our data and earlier studies [40, 52]. Therefore, we propose that these genes can be viewed as robust indicators of cellular hypoxia on mRNA level. A plethora of data suggests that, for tissue engineering, being able to improve COL2A1 expression may be more relevant than controlling AGC1. Since COL2A1 can be regarded as the prime marker for redifferentiation, with SOX9 driving its expression [60], we verified the induction of both key factors on protein level.

TGF-β ligands were already described as secreted polypeptides capable of inducing collagen production in general [61] and in cartilage in particular [25, 26]. We found a significant and selective upregulation of specifically the TGF-β2 isoform, which was verified on protein expression level (figure 2A and 2B). This led us to postulate that the endogenously produced TGF-β2 isoform is critically involved in the hypoxia-driven induction of COL2a1. Exogenous TGF-β2 is known to upregulate COL2a1 in redifferentiating chondrocytes [26, 57] and its expression has also been shown to be hypoxia-driven in other cells [13]. Subjecting human chondrocytes to 5% pO2, instead of 1% pO2, did not significantly alter

the expression of TGF-β2 (data not shown), which suggests a potential contribution of hypoxia inducible factors (HIF) to hypoxic COL2a1 production. The promoter regions of the TGF-β isoforms are appreciably different from each other [11] and characterization of the 5' UTR of the human TGF-β genes also revealed that TGF-β2 has the longest regulatory region of all isoforms to regulate its transcription and translation [11]. TGF-β2 was shown to auto-induce its own mRNA after just 3 hours [62]. Of note, blocking HIF-1a can also reduce TGF-β stimulated collagen production [63]. TGF-β2 is also different from the other two isoforms in that it needs betaglycan for signaling. TGF-β/Smad3 signaling is then able to inhibit terminal hypertrophic differentiation of chondrocytes [64]. Even low concentration of 1ng/mL of TGF-β can quickly upregulate its own receptors. In our study, protein levels of TGF-□2 were also increased under hypoxia over two-fold (figure 2B). Since low concentrations already have biological relevance, this increase can cause further downstream effects.

To further shed light on the hypoxia-mediated TGF-β signaling, we also screened the expression of relevant TGF-β receptors. Hypoxic culture significant increased expression levels of both type I receptors that are involved in TGF-β signaling in chondrocytes (ALK1 and ALK5 [30, 31]), but did not alter the expression of TGFBR2 or BGCAN and EGN (figure 3). We cannot exclude that post-transcriptional regulation affected receptor protein levels differently as we were not able to investigate this aspect; this will be addressed in future studies. A previous study also reported upregulation of one type I receptor under short term hypoxia (5% pO2 [15]), but only looked at ALK5. While BGCAN favors TGF-β2-mediated signaling, EGN regulates TGF-β receptor endocytosis and inhibits TGF-β signaling [65]. TGFBR2B (TbetaRII-B), a splice variant of TGFBR2, is also prominently expressed by osteoarthritic human chondrocytes and can form heteromeric complexes with all type I, II and III receptors and can thereby enhance TGF-β signaling in chondrocytes when being overexpressed [66]. However, expression of this variant is another unexplored aspect that may have contributed to the upregulation of COL2 levels under hypoxia. Interestingly, it is the ratio between the two type I receptors that is suggested to dictate the TGF-β response

in chondrocytes [30, 31]. Although hypoxia upregulated both ALK1 and ALK5, post transcriptional effects of hypoxia on either type I receptor might shift that balance on cell signaling level. Additionally, EGN, which binds TGF-β1 and -β3, but not TGF-β2 [67, 68], can inhibit the ALK5-mediated Smad2 phosphorylation (Smad2-P) in human chondrocytes [69]. Interestingly, hypoxia non-significantly decreased EGN expression, which would be in line with an ALK5-Smad2-P dominance between these two signaling routes. Future experiments should look into phosphorylation of Smads in this model to elucidate the active signaling routes involved.

Next, we employed RNAi to confirm a crucial contribution of TGF-β2 to the hypoxia-induced COL2 production. Upon confirmation of the isoform specificity of the anti-TGF-β2 shRNAs (figure 4A), we showed that, in primary human chondrocytes, severely hypoxic conditions (1% pO2) alone only just non-significantly upregulated COL2 mRNA expression in non-targeting controls (CTRL, figure 4B). Addition of exogenous TGF-β2 to these hypoxic cells, again prominently and synergistically, increased COL2 expression. Non-transient lentiviral anti-TGF-β2 shRNA expression significantly suppressed this induction in COL2 expression to below that of the non-targeting control. This is in line with a cell signaling model in which hypoxic conditions induce TGF-β2 synthesis in order to maintain a autoregulatory loop that induces COL2 expression in these cells. However, in cells which are actively expressing anti-TGF-β2 shRNAs, the hypoxia-induced nascent TGF-β2 mRNAs will be readily degraded. In these cells any positive contribution of TGF-β2 signaling to the COL2 expression is inhibited and cannot reach the same level as in the untreated hypoxic cells. On the other hand, as the knockdown is never 100% efficient, the residual TGF-β2 mRNAs in these cells may be sufficient to induce COL2 expression to just above normoxic control levels (figure 4B).

We further inhibited ALK5 to elucidate its role during the TGF-β2-mediated hypoxic induction of COL2 synthesis in human chondrocytes: while hypoxia induced COL2, ALK5i suppressed it (figure 4C). In chondrocytes, TGF-β signaling promotes HIF1A stabilization via ALK5 [70], which would establish a positive feedback loop between hypoxia and TGF-β signaling.

When demonstrating the hypoxia- and TGF-β2-dependency of COL2 expression, we further used PAI-1 as an internal control: its expression was recently shown to depend on both, TGF-β and HIF1A, in other cells [71]. Also, induction of PAI-1 crucially depends on SMAD3 and SMAD4 binding [47]. Exposure of HUVEC cells to 1% pO2 resulted in SMAD binding to the -77 to -40 bp region ("CAGA box") in the TGF-□2 promoter and increased TGF-β2 mRNA levels as compared to normoxic controls (20% pO2). These authors used a 3TP-lux reporter, a Smad2/3-responsive construct [72] similar to the Smad3-responsive CAGA12-lux reporter construct [73], which is basically derived from the minimal SREs of the PAI-1 promoter, to show the SMAD-dependency of TGF-□2 signaling in their system. Also here, TGF-β2 mRNA was the only specifically induced TGF-β isoform [13]. Finnson [30] showed that COL2 and PAI-1 is stimulated by exogenous TGF-β1 in chondrocytes. It has also been shown in hepatocytes that the HRE site in the PAI-1 promoter could be a target for CREB binding to mediate PAI-1 gene induction [74, 75]. Interestingly, TGF-β2 is also the only isoform with multiple, hypoxia-sensitive, CRE elements within 400 nucleotides upstream of its P1 promoter [11]. Although TGF-β2 is structurally similar to TGF-β1, their biological responses may differ cell type dependently. Interestingly, initial data suggest that cartilaginous effects of TGF-β at low concentration (1 ng/mL) are especially dependent on low oxygenation levels below 5% [55].

Recently, Finnson et al. [30] also showed that endoglin enhanced TGF-β1-induced Smad1/5 phosphorylation, but inhibits TGF-β-induced Smad2 phosphorylation, and Smad3-driven transcriptional COL2 and PAI-1 activation in chondrocytes. Our data support the notion that hypoxia induces the ALK5-SMAD2/3 pathway rather than ALK1-Smad1/5 signaling. We further used suppression of inhibitor of differentiation 1 (Id1), a downstream target of the BMPR2 pathway [76] to show the specificity of ALK5i: ID1 expression is not induced by hypoxia and not affected by ALK5i. ID-1 has been shown to be an ALK1 downstream target in endothelial cells [48]. Interesting here is that ALK1 also stimulates MMP13 expression and we found a (non-significant) trend towards down regulation in MMP13 mRNA levels. The ID-1/PAI-1 ratio also increases with OA progression (i.e. cartilage degradation) and that this is in line with a shift from ALK5

to predominant ALK1 signaling [31]. From figure 4C it is apparent that hypoxia increases the PAI-1/ID-1 ratio. We propose that this indicates a hypoxia-mediated shift in TGFβ signaling in favor of anabolic ALK5 signaling and ECM production. In addition, the Sp1 /Sp3 ratio is important for COL2A1 expression by chondrocytes, but was not studied by us. The levels of both transcription factors decrease with dedifferentiation [77] and Sp1 overexpression increases COL2A1 expression. TGF-β/Smad3 signals are essential for repressing articular chondrocyte differentiation. Without these inhibition signals, chondrocytes break quiescent state and undergo terminal differentiation [78]. It is interesting that Sp1 has been reported to be down-regulated by TGF-β1 [41]; it would be interesting to see if this also holds for TGF-β2 as our data would suggest the opposite.

In summary, our findings strongly suggest that the hypoxia-induced TGF-β2 expression in human articular chondrocytes in vitro is responsible for the subsequent induction of type II collagen by these cells. We further provide data supporting the notion that ALK5 is critically involved in this process. This study is the first to suggest that hypoxic culture conditions for cartilage tissue engineering may benefit from an auto-regulatory TGF-β signaling loop by the chondrocytes.

Acknowledgements

This research was funded by the Dutch Program Tissue Engineering (DPTE, Project No. RGT.6738) and the START initiative of the RWTH Aachen.

References

1. Silver, I.A., Measurement of pH and ionic composition of pericellular sites. Philos Trans R Soc Lond B Biol Sci, 1975. 271(912): p. 261-72.
2. Falchuk, K.H., E.J. Goetzl, and J.P. Kulka, Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. Am J Med, 1970. 49(2): p. 223-31.
3. Lund-Olesen, K., Oxygen tension in synovial fluids. Arthritis Rheum, 1970. 13(6): p. 769-76.
4. Treuhaft, P.S. and M.C. DJ, Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. Arthritis Rheum, 1971. 14(4): p. 475-84.
5. Grimshaw, M.J. and R.M. Mason, Bovine articular chondrocyte function in vitro depends upon oxygen tension. Osteoarthritis Cartilage, 2000. 8(5): p. 386-92.
6. Malda, J., et a., Cartilage tissue engineering: controversy in the effect of oxygen. Crit Rev Biotechnol, 2003. 23(3): p. 175-94.
7. Grimshaw, M.J. and R.M. Mason, Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension. Osteoarthritis Cartilage, 2001. 9(4): p. 357-64.
8. Coyle, C.H., N.J. Izzo, and C.R. Chu, Sustained hypoxia enhances chondrocyte matrix synthesis. J Orthop Res, 2009. 27(6): p. 793-9.
9. Domm, C., et al., Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. Tissue Eng, 2004. 10(11-12): p. 1796-805.
10. Miyazawa, K., et al., Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells, 2002. 7(12): p. 1191-204.
11. Roberts, A.B. and M.B. Sporn, Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. Mol Reprod Dev, 1992. 32(2): p. 91-8.
12. Sanford, L.P., et al., TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development, 1997. 124(13): p. 2659-70.
13. Akman, H.O., et al., Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. Blood, 2001.

98(12): p. 3324-31.
14. Falanga, V., et al., Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. J Invest Dermatol, 1991. 97(4): p. 634-7.
15. Martin, G., et al., Effect of hypoxia and reoxygenation on gene expression and response to interleukin-1 in cultured articular chondrocytes. Arthritis Rheum, 2004. 50(11): p. 3549-60.
16. Lyons, R.M., J. Keski-Oja, and H.L. Moses, Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. J Cell Biol, 1988. 106(5): p. 1659-65.
17. Jullien, P., T.M. Berg, and D.A. Lawrence, Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. Int J Cancer, 1989. 43(5): p. 886-91.
18. Yee, J.A., et al., Plasminogen-dependent activation of latent transforming growth factor beta (TGF beta) by growing cultures of osteoblast-like cells. J Cell Physiol, 1993. 157(3): p. 528-34.
19. Annes, J.P., J.S. Munger, and D.B. Rifkin, Making sense of latent TGFbeta activation. J Cell Sci, 2003. 116(Pt 2): p. 217-24.
20. van der Kraan, P.M., et al., TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. Osteoarthritis Cartilage, 2009. 17(12): p. 1539-45.
21. O'Connor, W.J., et al., The use of growth factors in cartilage repair. Orthop Clin North Am, 2000. 31(3): p. 399-410.
22. Demoor-Fossard, M., et al., Differential effects of interleukin-1 and transforming growth factor beta on the synthesis of small proteoglycans by rabbit articular chondrocytes cultured in alginate beads as compared to monolayers. Mol Cell Biochem, 1999. 199(1-2): p. 69-80.
23. Vivien, D., et al., Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes. J Cell Physiol, 1990. 143(3): p. 534-45.
24. van der Kraan, P.M., et al., Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage, 2002. 10(8): p. 631-7.
25. van Osch, G.J., et al., Growth factors in cartilage tissue engineering. Biorheology, 2002. 39(1-2): p. 215-20.
26. Yaeger, P.C., et al., Synergistic action of transforming growth factor-

beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp Cell Res, 1997. 237(2): p. 318-25.
27. Okazaki, R., et al., Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. Ann Rheum Dis, 1996. 55(3): p. 181-6.
28. Leonard, M.O., et al., Hypoxia selectively activates the CREB family of transcription factors in the in vivo lung. Am J Respir Crit Care Med, 2008. 178(9): p. 977-83.
29. Heldin, C.H., K. Miyazono, and P. ten Dijke, TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature, 1997. 390(6659): p. 465-71.
30. Finnson, K.W., et al., ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J Bone Miner Res, 2008. 23(6): p. 896-906.
31. Blaney Davidson, E.N., et al., Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol, 2009. 182(12): p. 7937-45.
32. Das, R.H., et al., Effects of individual control of pH and hypoxia in chondrocyte culture. J Orthop Res, 2010. 28(4): p. 537-45.
33. Uitterlinden, E.J., et al., Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. Osteoarthritis Cartilage, 2007. 15(11): p. 1267-74.
34. Uitterlinden, E.J., et al., Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage, 2006. 14(3): p. 250-7.
35. Bustin, S.A., et al., The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem, 2009. 55(4): p. 611-22.
36. van der Windt, A.E., et al., Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro. Arthritis Res Ther, 2010. 12(3): p. R100.
37. Foldager, C.B., et al., Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. BMC Mol Biol, 2009. 10: p. 94.
38. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.

Methods, 2001. 25(4): p. 402-8.
39. Das, R.H., et al., In vitro expansion affects the response of chondrocytes to mechanical stimulation. Osteoarthritis Cartilage, 2008. 16(3): p. 385-91.
40. Lafont, J.E., et al., Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. J Biol Chem, 2008. 283(8): p. 4778-86.
41. Bauge, C., et al., Modulation of transforming growth factor beta signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of Sp1 in both early and late response cells to transforming growth factor beta. Arthritis Res Ther, 2011. 13(1): p. R23.
42. van der Windt, A.E., et al., Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. Arthritis Rheum, 2012. 64(6): p. 1929-39.
43. Caron, M.M., et al., Osmolarity determines the in vitro chondrogenic differentiation capacity of progenitor cells via nuclear factor of activated T-cells 5. Bone, 2013. 53(1): p. 94-102.
44. Siebelt, M., et al., Hsp90 inhibition protects against biomechanically induced osteoarthritis in rats. Arthritis Rheum, 2013. 65(8): p. 2102-12.
45. Grygielko, E.T., et al., Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-beta type I receptor kinase in puromycin-induced nephritis. J Pharmacol Exp Ther, 2005. 313(3): p. 943-51.
46. Goumans, M.J., et al., Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J, 2002. 21(7): p. 1743-53.
47. Dennler, S., et al., Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J, 1998. 17(11): p. 3091-100.
48. Lebrin, F., et al., Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J, 2004. 23(20): p. 4018-28.
49. Fujita, N., et al., Expression of prolyl hydroxylases (PHDs) is selectively controlled by HIF-1 and HIF-2 proteins in nucleus pulposus cells of the intervertebral disc: distinct roles of PHD2 and PHD3 proteins in controlling HIF-1alpha activity in hypoxia. J Biol Chem, 2012. 287(20): p. 16975-86.
50. Hashimoto, K., et al., Hypoxia-induced hyaluronan synthesis by

articular chondrocytes: the role of nitric oxide. Inflamm Res, 2006. 55(2): p. 72-7.
51. Lee, J.J., et al., Hypoxia activates the cyclooxygenase-2-prostaglandin E synthase axis. Carcinogenesis, 2010. 31(3): p. 427-34.
52. Markway, B.D., H. Cho, and B. Johnstone, Hypoxia promotes redifferentiation and suppresses markers of hypertrophy and degeneration in both healthy and osteoarthritic chondrocytes. Arthritis Res Ther, 2013. 15(4): p. R92.
53. Lefebvre, V., et al., SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol, 1997. 17(4): p. 2336-46.
54. Stokes, D.G., et al., Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. Biochem J, 2001. 360(Pt 2): p. 461-70.
55. Murphy, C.L., et al., Hypoxia. HIF-mediated articular chondrocyte function: prospects for cartilage repair. Arthritis Res Ther, 2009. 11(1): p. 213.
56. D'Angelo, M., et al., MMP-13 is induced during chondrocyte hypertrophy. J Cell Biochem, 2000. 77(4): p. 678-93.
57. Van Osch, G.J., et al., Chondrogenic potential of in vitro multiplied rabbit perichondrium cells cultured in alginate beads in defined medium. Tissue Eng, 2000. 6(4): p. 321-30.
58. Walmsley, S.R., et al., Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. J Clin Invest, 2011. 121(3): p. 1053-63.
59. Zhang, C., et al., Regulation of VEGF expression by HIF-1alpha in the femoral head cartilage following ischemia osteonecrosis. Sci Rep, 2012. 2: p. 650.
60. Bell, D.M., et al., SOX9 directly regulates the type-II collagen gene. Nat Genet, 1997. 16(2): p. 174-8.
61. Roberts, A.B., et al., Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A, 1986. 83(12): p. 4167-71.
62. Miura, T. and K. Shiota, TGFbeta2 acts as an "activator" molecule in reaction-diffusion model and is involved in cell sorting phenomenon in

mouse limb micromass culture. Dev Dyn, 2000. 217(3): p. 241-9.
63. Basu, R.K., et al., Interdependence of HIF-1alpha and TGF-beta/Smad3 signaling in normoxic and hypoxic renal epithelial cell collagen expression. Am J Physiol Renal Physiol, 2011. 300(4): p. F898-905.
64. Grimaud, E., D. Heymann, and F. Redini, Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders. Cytokine Growth Factor Rev, 2002. 13(3): p. 241-57.
65. Bizet, A.A., et al., The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. Biochim Biophys Acta, 2011. 1813(5): p. 742-53.
66. Parker, W.L., et al., Expression and function of TbetaRII-B, a variant of the type II TGF-beta receptor, in human chondrocytes. Osteoarthritis Cartilage, 2007. 15(4): p. 442-53.
67. Brown, C.B., et al., Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science, 1999. 283(5410): p. 2080-2.
68. Sankar, S., et al., Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2. J Biol Chem, 1995. 270(22): p. 13567-72.
69. Finnson, K.W., et al., Endoglin differentially regulates TGF-beta-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes. Osteoarthritis Cartilage, 2010. 18(11): p. 1518-27.
70. Estrada, K.D., et al., Smad7 regulates terminal maturation of chondrocytes in the growth plate. Dev Biol, 2013. 382(2): p. 375-84.
71. Ueno, M., et al., Hypoxia-inducible factor-1alpha mediates TGF-beta-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol, 2011. 300(5): p. L740-52.
72. Wrana, J.L., et al., TGF beta signals through a heteromeric protein kinase receptor complex. Cell, 1992. 71(6): p. 1003-14.
73. Dennler, S., S. Huet, and J.M. Gauthier, A short amino-acid sequence in MH1 domain is responsible for functional differences between Smad2 and Smad3. Oncogene, 1999. 18(8): p. 1643-8.
74. Kvietikova, I., et al., The hypoxia-inducible factor-1 DNA

recognition site is cAMP-responsive. Kidney Int, 1997. 51(2): p. 564-6.
75. Dimova, E.Y., M.M. Jakubowska, and T. Kietzmann, CREB binding to the hypoxia-inducible factor-1 responsive elements in the plasminogen activator inhibitor-1 promoter mediates the glucagon effect. Thromb Haemost, 2007. 98(2): p. 296-303.
76. Wu, X., et al., Hypoxia regulates bone morphogenetic protein signaling through C-terminal-binding protein 1. Circ Res, 2006. 99(3): p. 240-7.
77. Ghayor, C., et al., Sp3 represses the Sp1-mediated transactivation of the human COL2A1 gene in primary and de-differentiated chondrocytes. J Biol Chem, 2001. 276(40): p. 36881-95.
78. Yang, X., et al., TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol, 2001. 153(1): p. 35-46.

5

Chapter 5

Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro

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Published as van der Windt et al., Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro, Arthritis Res Ther. 2010; 12(3): R100

Summary

Chondrocytes experience a hypertonic environment compared with plasma (280 mOsm) due to the high fixed negative charge density of cartilage. Standard isolation of chondrocytes removes their hypertonic matrix, exposing them to nonphysiological conditions. During in vitro expansion, chondrocytes quickly lose their specialized phenotype, making them inappropriate for cell-based regenerative strategies. We aimed to elucidate the effects of tonicity during isolation and in vitro expansion on chondrocyte phenotype.

Human articular chondrocytes were isolated and subsequently expanded at control tonicity (280 mOsm) or at moderately elevated, physiological tonicity (380 mOsm). The effects of physiological tonicity on chondrocyte proliferation and chondrogenic marker expression were evaluated. The role of Tonicity-responsive Enhancer Binding Protein in response to physiological tonicity was investigated using nuclear factor of activated T-cells 5 (NFAT5) RNA interference.

Moderately elevated, physiological tonicity (380 mOsm) did not affect chondrocyte proliferation, while higher tonicities inhibited proliferation and diminished cell viability. Physiological tonicity improved expression of chondrogenic markers and NFAT5 and its target genes, while suppressing dedifferentiation marker collagen type I and improving type II/type I expression ratios >100-fold. Effects of physiological tonicity were similar in osteoarthritic and normal (nonosteoarthritic) chondrocytes, indicating a disease-independent mechanism. NFAT5 RNA interference abolished tonicity-mediated effects and revealed that NFAT5 positively regulates collagen type II expression, while suppressing type I.

Physiological tonicity provides a simple, yet effective, means to improve phenotypical characteristics during cytokine-free isolation and in vitro expansion of human articular chondrocytes. Our findings will lead to the development of improved cell-based repair strategies for chondral lesions and provides important insights into mechanisms underlying osteoarthritic progression.

Introduction

Hyaline articular cartilage is a connective tissue covering the ends of bones in joints and is composed of specialized cells, chondrocytes that produce a large amount of extracellular matrix. This matrix is crucial for the unique biomechanical properties of this tissue and is composed of a collagen fiber network, providing tensile strength and flexibility, and abundant ground matrix rich in proteoglycans [1]. The glycosaminoglycan (GAG) side chains of the proteoglycans are sulfated and responsible for a characteristic high fixed negative charge density [2], which binds mobile cations (mainly sodium). This binding determines the physiological tonicity (that is, osmotic pressure) of the extracellular fluid around chondrocytes in vivo, but the tonicity indirectly also largely depends on the quality of the collagen network. Extracellular tonicity in healthy cartilage ranges between 350 and 480 mOsm [3, 4]. In vivo, tonicity of the extracellular fluid is dynamic and changes due to alterations in matrix hydration [5]. During cartilage degeneration (that is, in osteoarthritis (OA)), the collagen matrix degrades and the GAG concentration diminishes, resulting in a severity-dependent decreased tonicity of between 280 and 350 mOsm [3, 6]. Currently, chondrocyte isolation and in vitro expansion culture are performed in medium of nonphysiological tonicity (270 ± 20 mOsm). Several studies have already shown that chondrocytes are tonicity responsive [7-9] and react with changes in matrix synthesis [4, 8, 10, 11], but focused on aggrecan (AGC1) core protein mRNA levels, AGC1 promoter activity and GAG production.

Molecular mechanisms involved in the hypertonic response of human articular chondrocytes (HACs) are poorly understood. Hypertonicity perturbs cells by causing osmotic efflux of water, resulting in cell shrinkage [12, 13]. Cells react by a rapid uptake of ions, which increase cellular ionic strength [14] with potentially detrimental effects [15-17]. The initial, rapid response is the activation of transporters that exchange these ions for compatible osmolytes [16, 18]. This process is controlled by Tonicity-responsive Enhancer Binding Protein (TonEBP/NFAT5), which mediates transcriptional activation of these transporters [16]. Nuclear factor of activated T-cells 5 (NFAT5) is a member of the Rel family of transcription

factors [19] and targets sodium/myo-inositol cotransporter (SMIT) [20, 21], sodium/chloride-coupled acid transporters (BGT1/SLC6A12) [20], aquaporin channels (AQP1 and AQP2) [22], and calcium-binding proteins (S100A4) [23-25]. Upon hypertonic stress, transcription of NFAT5 itself is upregulated in several cell types [26-28], but the tonicity threshold and cell signalling pathways required to activate NFAT5 may be cell type specific [29]. Nothing is currently known about the expression or function of NFAT5 in HACs.

Chondral lesions from, for example, trauma or overuse, can cause joint pain, immobility and eventually OA. The associated high prevalence - 60% of all patients undergoing knee arthroscopy are diagnosed with a chondral lesion [30] - and loss of quality of life makes cartilage damage a major personal and economical burden. Treatment options for chondral lesions are limited, and autologous chondrocyte implantation is the currently most developed hyaline repair technique for the knee [31]. Characterized chondrocyte implantation, employing phenotypical prescreening prior to implantation, has recently improved structural repair [32].

Chondrocyte dedifferentiation during in vitro expansion for autologous chondrocyte implantation is detrimental; but almost inevitably in standard monolayer culture, spherical chondrocytes will gradually convert into fibroblast-like cells [33, 34]. This morphological change is accompanied by a shift in collagen expression towards less collagen type II (COL2) and more collagen type I (COL1) [34, 35]. Consequently, dedifferentiated chondrocytes produce fibrocartilage in vivo, with an extracellular matrix of inferior biomechanical properties due to higher collagen (especially type I) content and less proteoglycans compared with native hyaline cartilage [36]. Three-dimensional culture systems can partially prevent dedifferentiation, but are labor intensive and essentially impair propagation. Chondrocyte dedifferentiation might also play a role in the pathogenesis of OA, as the ability of aging chondrocytes to produce and repair the extracellular matrix is compromised [37] and as COL1 is shown to be present in chondrocyte clusters in fibrillated areas of late-stage OA cartilage while it is absent in healthy cartilage [38].

In the present article we report that physiological tonicity (380 mOsm) during isolation and monolayer expansion can suppress chondrocyte dedifferentiation and that expression of the extracelluar matrix components collagen type I and collagen type II as well as aggrecan is NFAT5 dependent. We further show that NFAT5 contributes to the differential regulation of both collagen types. This study provides a simple, yet novel and effective, means to improve cell-based repair strategies for chondral lesions and contribute to our understanding of OA progression.

Materials and Methods

Cartilage and chondrocyte isolation

After informed consent was obtained, human articular cartilage was explanted from macroscopically normal areas of the femoral condyles and tibial plateau of nine patients undergoing total knee replacement surgery for OA (medical ethical approval MEC2004-322). In addition to preparation of cartilage explants and isolation of HACs under standard conditions (DMEM, 280 mOsm) as described by Das and colleagues [39], medium tonicity was also adjusted to 380 mOsm, 480 mOsm or 580 mOsm by addition of sterile NaCl. Enzymatic digestion, removal of undigested fragments and subsequent chondrocyte culture were all reported earlier [39]. The 280 mOsm and 380 mOsm isolations were also performed with cartilage obtained from the femoral condyles and tibial plateau of two non-OA donors (further referred to as normal donors) undergoing above-knee amputation surgery after trauma.

Chondrocyte proliferation and DNA measurements

Primary (P0), passage 1 (P1), passage 2 (P2) and passage 3 (P3) HACs were monolayer expanded in medium corresponding to their isolation tonicity (280 mOsm, 380 mOsm, 480 mOsm or 580 mOsm), with an initial seeding density of 6,000 cells/cm2. Cells were harvested daily for cell counts and DNA assay between days 2 and 6. Experiments were performed in duplicate from three OA donors (n = 6). At each passage, growth curves were established by cell counts using Trypan Blue (catalogue number T8154; Sigma-Aldrich, St. Louis, MO, USA) and DNA quantification. DNA measurements were performed according to Karsten and Wollenberger

[40] with slight modifications [41]. Doubling times within each passage were calculated from the trend line of the exponential growth phase using the equation:

$$y=x(0)\exp(kx(t)$$

$$k=\ln 2/T$$

where k is the growth constant and T is the doubling time.

Chondrocyte expansion

Primary HACs were cultured for expansion in monolayers at a seeding density of 7,500 cells/cm2 in medium corresponding to their isolation tonicity (280 mOsm, 380 mOsm, 480 mOsm or 580 mOsm). P0 cells to P3 cells were seeded in high-density monolayers (20,000 cells/cm2) and were cultured for an additional 5 days and 7 days before analysis of mRNA (quantitative RT-PCR) and protein expression (Western blotting), respectively. Experiments were performed in triplicate from four OA donors (n = 12). Additional experiments were performed in triplicate from two healthy donors (n = 6) to investigate whether the hypertonic stress response is specific for pathologically altered cells. To exclude sodium-specific or chloride-specific effects, we performed experiments using N-methyl-d-glucamine chloride (NMDG-Cl) or sucrose to adjust the medium tonicity to 380 mOsm.

Lentiviral NFAT5 gene knockdown

We used lentiviral vectors for nontransient shRNA-mediated gene silencing in primary chondrocytes [42]. BamHI/MunI restriction fragments of the parental pLKO.1-puro vector - each containing the U6 promotor and one out of five different, sequence-verified antihuman NFAT5 shDNAs (MISSION shRNA library [43]) -were subcloned into corresponding restriction sites of recipient vector pRRL.PPT.PGK.GFPpre. This vector was kindly provided by L Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) [44, 45] and was optimized by A Schambach (Department of Experimental Hematology, Hannover Medical School, Hannover, Germany) [46] to express enhanced green fluorescent protein

(eGFP) from the phosphoglycerate kinase promoter. Lentiviral particles were produced in HEK293T cells by transient transfection using a calcium phosphate protocol [47]. Cells transduced with a lentiviral vector lacking the NFAT5-specific shRNA expression cassette served as controls. All cells were grown in monolayers. TRCN0000020020 was identified as the best performing anti-NFAT5 shRNA clone by quantitative PCR-based knockdown efficiency determination, and was used in subsequent experiments.
P1 OA HACs from two donors were seeded (15,000 cells/cm2) and cultured for 4 days in control medium (280 mOsm). Three hours prior to transduction, cells were deprived of antibiotics, and then were transduced for□± 18 hours, refreshed with control medium with antibiotics and cultured for an additional 4 days before harvesting for fluorescence-activated cell sorting (FACS) analyses. Cells were resuspended in PBS with 10% FCS and antibiotics, and were washed. Cells were collected and stained with Hoechst 33258 (1 mg/ml; Molecular Probes/Invitrogen Corp., Carlsbad, CA, USA) to discriminate between dead cells and live cells. FACS was performed on the FACSAria (Becton Dickinson BV, Breda, The Nederlands), and eGFP-expressing cells were collected (>50%, multiplicity of infection ~1) and reanalyzed for purity (>95%) using Cell Quest Pro Software (Becton Dickinson Biosciences BV, Breda, The Nederlands).

The eGFP-expressing populations were seeded (10,000 cells/cm2) and cultured in control medium up to 80% confluency. Cells were then switched to medium of 380 mOsm or were kept on control medium for 24 hours prior to RNA analysis.

RNA expression analysis
RNA isolation, purification, quantification and cDNA synthesis are described elsewhere [48]. Expression levels of AGC1, SOX9 and COL2 were studied as chondrogenic markers, while COL1 was studied as a dedifferentiation marker [34, 35, 49, 50]. Quantitative PCR assays for COL2, SOX9, AGC1 and COL1 have been reported earlier [51].
To quantify expression of NFAT5 and its target genes, the following primers were tested for similar amplification efficiency and specificity according to Das and colleagues [39], and were used as 20 µl SYBRR

Green I reactions: HsNFAT5_Fw, GGGTCAAACGACGAGATTGTG and HsNFAT5_Rv, TTGTCCGTGGTAAGCTGAGAA; HsS100A4_Fw, GTCCACCTTCCACAAGTAC TCG and HsS100A4_Rv, TCATCTGTCCTTTTCCCCAAG; and HsSLC6A12_Fw, ACACAGAGCATTGCACGGACT and HsSLC6A12_Rv, CCAGAACTCGTC TCTCCCAGAA. Data were normalized to an index of three reference genes (GAPDH, UBC, HPRT1) that were pre-evaluated to be stably expressed across samples [39]. Relative expression was calculated according to the 2-ΔCT method [52].

Western blot analysis

Cells seeded at high densities were washed twice with PBS and were lysed in RIPA buffer [53] with addition of protease inhibitors. The total protein concentration was quantified by the bicinchoninic acid assay according to the manufacturer's protocol (#23225; Thermo Fisher Sci., Rockford, IL, USA). Aliquots (10 µg) were subjected to 10% SDS-PAGE prior to electroblotting onto nitrocellulose membranes (Protran BA83; Schleicher & Schuell BV, s-Hertogenbosch, The Netherlands). Blots were blocked in 5% low-fat dry milk in 1× PBS, 0.05% v/v NP-40, were incubated with primary antibodies - anti-type II collagen and anti-type I collagen, both 1:100 (SouthernBiotech, Birmingham, Alabama, USA), or 1:10,000 anti-α-Tubulin (Sigma) - were washed, were incubated with secondary antibodies (both 1:1,000; Dako Cytomation, Heverlee, Belgium) and were chemiluminescently detected. Signals were quantified using ImageJ 1.42 software [54].

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were compared between groups by Kruskall-Wallis H test and post-hoc Mann-Whitney U test. Results represent the mean ± standard deviation, and $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered to indicate levels of statistically significant difference.

Results

Hypertonicity influences proliferation and survival of chondrocytes
We first determined the influence of tonicity on proliferation: OA HACs isolated at 580 mOsm hardly attached or proliferated (figure 1D), and 2 days after seeding no viable cells were recovered. At 280 mOsm, 380 mOsm and 480 mOsm, respectively, cells did adhere but increasing tonicity induced marked morphological changes: at 280 mOsm, cells appeared fibroblast-like, stretched out and flattened with long filopodia (figure 1A); while at 380 mOsm, cells were more sphere-shaped and had shorter filopodia (figure 1B). At 480 mOsm, cells showed few filopodia and appeared spherical (figure 1C). The differences in appearance remained throughout the dedifferentiation period (P0 to P3), but were most apparent at earlier passages.

Using cell counts and DNA assays, doubling times were calculated from growth curves established from each passage at three different tonicities (280 mOsm, 380 mOsm and 480 mOsm). Throughout dedifferentiation, OA HACs isolated at 480 mOsm showed severely inhibited proliferation compared with cells at 280 mOsm and 380 mOsm (Table 1). In contrast, doubling times of OA HACs at 280 mOsm and 380 mOsm never significantly differed (Table 1). All further experiments were therefore performed at 380 mOsm (as high tonicity condition) and compared with 280 mOsm (control condition).

TABLE 1
Proliferation of chondrocytes isolated and cultured at 280 mOsm, 380 mOsm and 480 mOsm

	Chondrocyte proliferation (%)			
Culture Condition	**Passage 0**	**Passage 1**	**Passage 2**	**Passage 3**
280 mOsm	100 (68±28 hours)	100 (89±54 hours)	100 (67±48 hours)	100 (57±11 hours)
380 mOsm	113±18	89±25	99±9	154±41
480 mOsm	675±405*	180±24*	168±28*	165±81*

Data presented as relative doubling times in percentage of cells cultured at 280 mOsm, mean ± standard deviation. The absolute doubling time ± standard deviation in hours is displayed in brackets. n = 6. mOsm, milliosmoles per kilogram of water. *$P < 0.05$.

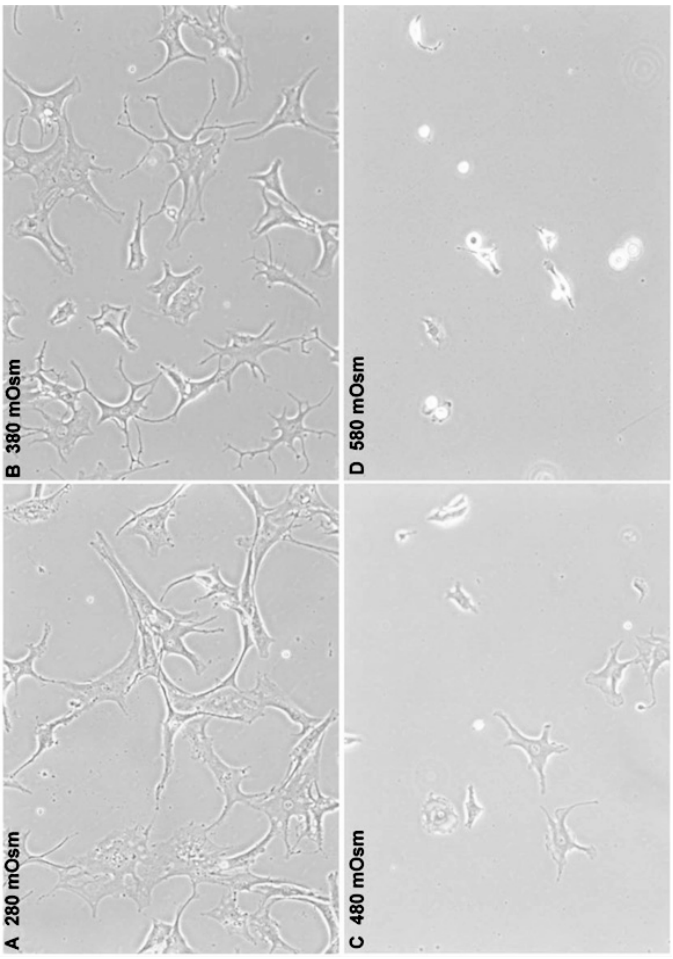

Figure 1: Hypertonic isolation and expansion of chondrocytes changes chondrocyte morphology. Representative images (200×) of chondrocytes cultured for 2 days at (a) 280 mOsm, (b) 380 mOsm, (c) 480 mOsm and (d) 580 mOsm.

Isolation and expansion of chondrocytes under hypertonic conditions improves their phenotype

Next, we set out to determine whether expansion culture in physiological tonicity improves the chondrocytic phenotype. Physiological tonicity (380 mOsm) during isolation and subsequent passaging of OA HACs significantly increased mRNA levels of both AGC1 (figure 2A) and SOX9 (figure 2B) at all passages. In expanded P3 chondrocytes in physiological culture, AGC1 levels were still higher than in unpassaged P0 chondrocytes cultured under the standard culture conditions (280 mOsm).

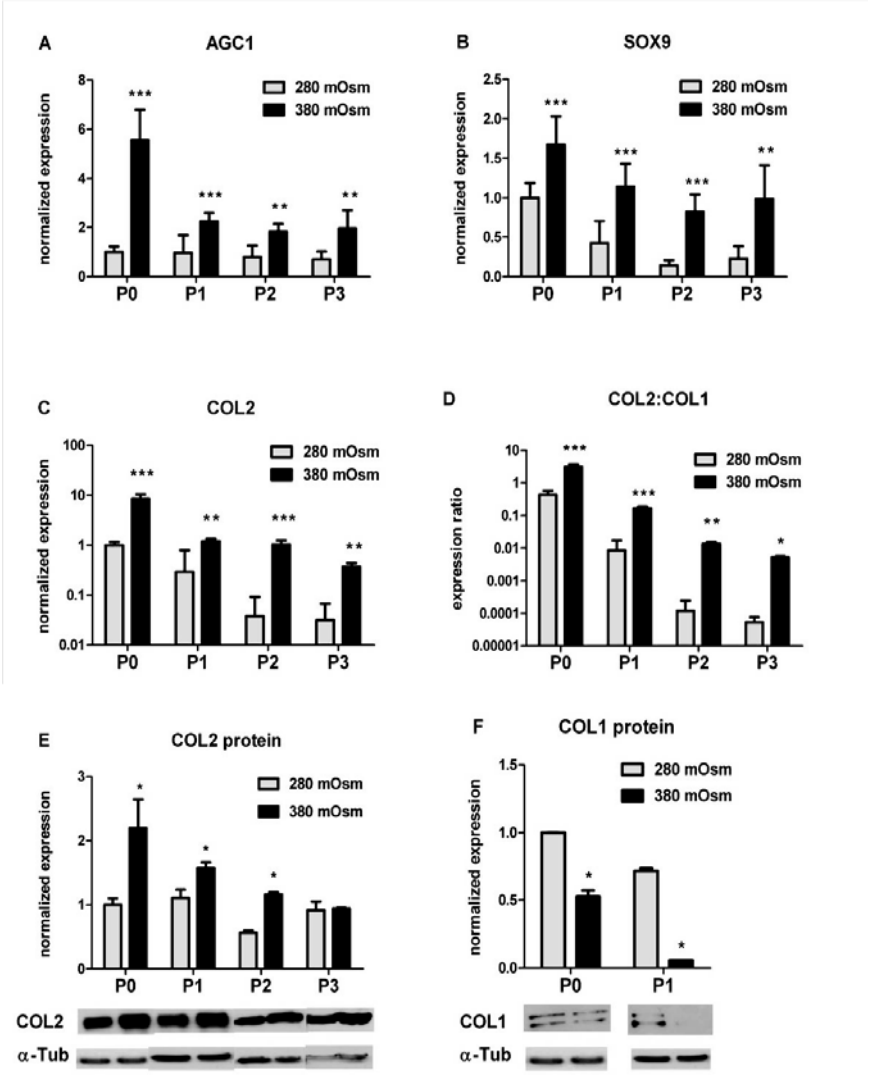

Figure 2: Hypertonic isolation and expansion increased marker gene expression in osteoarthritis human articular chondrocytes. Relative expression of (a) AGC1, (b) SOX9, (c) COL2 and (d) COL2:COL1 ratio in primary (P0) and passaged (P1 to P3) chondrocytes cultured at 380 mOsm compared with 280 mOsm. (e) COL2 protein expression and (f) COL1 protein expression in P0 and P1 osteoarthritis human articular chondrocytes. Protein levels normalized to α-tubulin. Data are mean ± standard deviation, n = 12. Differences from cells cultured at 280 mOsm are indicated: *P < 0.05, **P < 0.01 and ***P < 0.001.

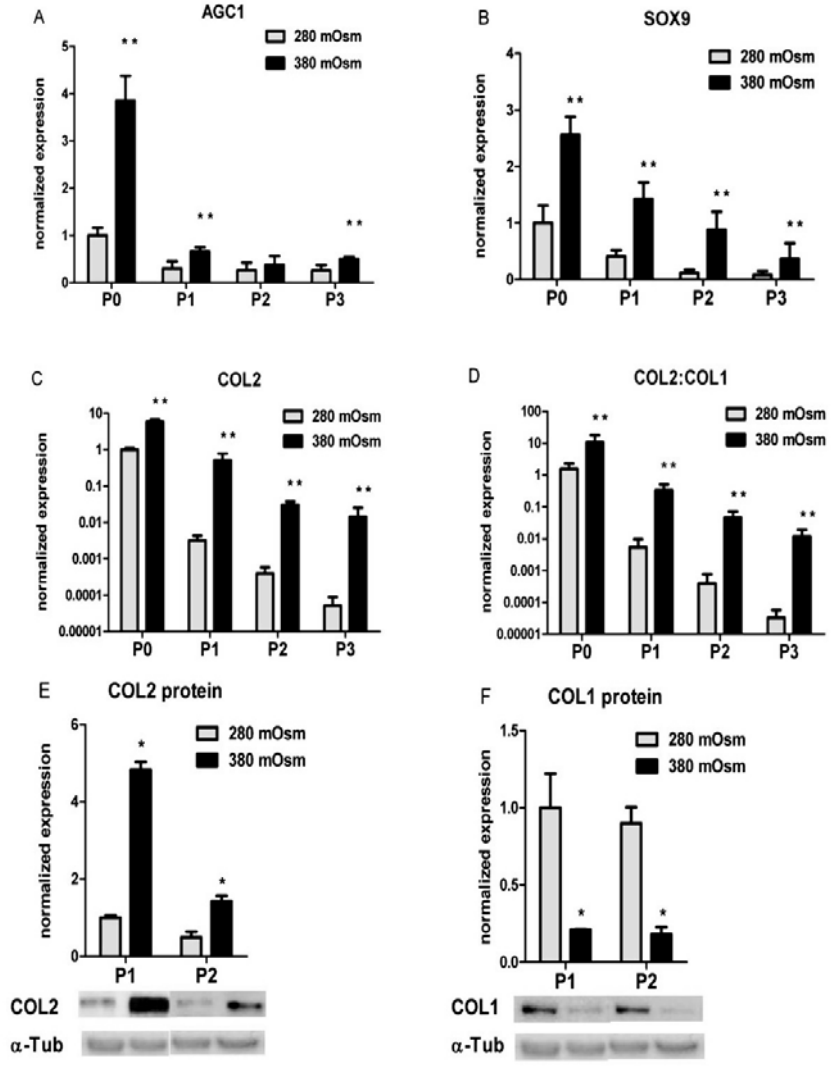

Figure 3: Hypertonic isolation and expansion increased chondrogenic marker expression in nonosteoarthritic human articular chondrocytes. Relative expression of (a) AGC1, (b) SOX9, (c) COL2 and (d) COL2:COL1 ratio in primary (P0) and passaged (P1 to P3) nonosteoarthritic human articular chondrocytes (NHACs) cultured at 380 mOsm compared with cells cultured at 280 mOsm. (e) COL2 protein expression and (f) COL1 protein expression in P1 and P2 NHACs, normalized to α-tubulin. Data are mean ± standard deviation, n = 6. Differences from 280 mOsm controls are indicated: *P < 0.05 and **P < 0.01.

Physiological tonicity also significantly upregulated COL2 levels from 8.5-fold in P0 to 11.6-fold in expanded P3 chondrocytes (figure 2C) compared with controls. In contrast, COL1 expression was significantly suppressed in physiological conditions throughout culture. Consequently, we found a significantly improved COL2/COL1 ratio during chondrocyte expansion (figure 2D), from sevenfold in P0 cells to 100-fold in expanded P3 cells. Physiological tonicity also upregulated COL2 protein expression (figure 2C): levels significantly increased (between 1.5-fold and 2.2-fold) in P0, P1 and P2 chondrocytes. In contrast, physiological tonicity significantly decreased COL1 protein expression (figure 2F), from twofold in P0 cells to 13-fold in P1 cells.

Physiological tonicity also significantly increased AGC1 (figure 3A) and SOX9 (figure 3B) mRNA levels in nonosteoarthritic human articular chondrocytes (NHACs). Furthermore, COL2 mRNA levels were significantly upregulated, from 5.8-fold in P0 cells to 270-fold in expanded P3 NHACs (figure 3C). As in OA HACs, hypertonicity also downregulated COL1 expression with increasing passage number in NHACs: the COL2/COL1 ratios increased during expansion (figure 3D), from 6.8- fold in P0 cells to 355-fold in expanded P3 cells. Correspondingly, COL2 protein levels increased under these conditions (4.8-fold in P1 cells and 2.9-fold in P2 cells), while the amount of COL1 diminished (by 4.7-fold in P1 cells and fivefold in P2 cells) (figure 3E, F).

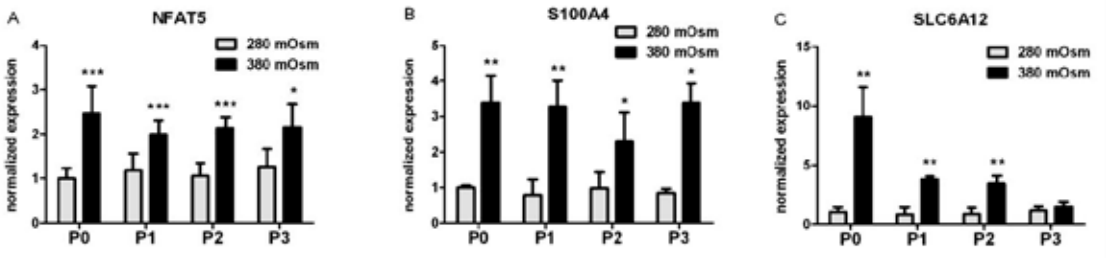

Figure 4: Hypertonic conditions activate nuclear factor of activated T-cells 5 in osteoarthritis human articular chondrocytes. Relative expression of (a) nuclear factor of activated T-cells 5 (NFAT5) and its target genes (b) S100A4 and (c) SLC6A12 in primary (P0) and passaged (P1 to P3) chondrocytes cultured at 380 mOsm compared with 280 mOsm. Data are mean ± standard deviation, n = 12. Differences are indicated: *P < 0.05, **P < 0.01 and ***P < 0.001.

Hypertonicity activates NFAT5 in human articular chondrocytes

Compared with 280 mOsm controls, NFAT5 mRNA levels were significantly increased in 380 mOsm OA HAC cultures (figure 4A), as was the expression of established NFAT5 target genes S100A4 (in all passages; figure 4B) and SLC6A12 (until P2; figure 4C). Similar effects were found in NHACs (data not shown).

NFAT5 knockdown inhibits hypertonicity-induced chondrogenic marker expression

Upon transduction, sorted eGFP-coexpressing OA HACs were switched to 380 mOsm for 24 hours. In controls not expressing NFAT5-specific shRNAs, an approximately twofold increase in NFAT5 mRNA levels was observed upon hypertonic stimulation (figure 4A, P1). In contrast, likewise challenged cells expressing anti-NFAT5 shRNAs showed an approximately 75% reduction in NFAT5 levels (figure 5A). Following NFAT5 knockdown, the NFAT5 targets S100A4 and SLC6A12 were also no longer hypertonically inducible: S100A4 expression decreased twofold and SLC6A12 was virtually undetectable upon NFAT5 RNAi (figure 5A), confirming a functional NFAT5 knockdown. At 380 mOsm, NFAT5 RNAi also downregulated chondrogenic markers: AGC1 by 80%, SOX9 by 32% and COL2 by 84%, as compared with non-RNAi controls (figure 5B). Interestingly, expression of COL1 increased after NFAT5 RNAi in OA HACs to ~300% of control levels (figure 5B).

Discussion

Isolation and expansion of adult HACs under physiological tonicity (380 mOsm) improves expression of chondrogenic markers on mRNA and protein levels. While other studies partially confirm that nonhuman chondrocytes respond to tonicity with altered aggrecan and SOX9 expression [4, 8, 10], we are reporting beneficial effects of isolating and expanding human normal and OA articular chondrocytes at physiological levels (380 mOsm). In addition, we also studied collagen type II expression, generally acknowledged to be the most important chondrogenic marker. As fibrocartilaginous collagen type I and hyaline collagen type II expression are differentially regulated in chondrocytes [34], analyzing the collagen

type II/type I expression ratios is informative of chondrogenic potential [51]. Interestingly, NFAT5 seems to be crucially involved in this differential regulation upon hypertonic challenge: it positively regulates collagen type II, while suppressing collagen type I (figure 5B). Fibrocartilage, occurring in areas subject to frequent stress like intervertebral discs and tendon attachment sites, is more rich in collagen type I than is hyaline cartilage [55]. Tonicity may thus provide a simple means to manipulate expression of these two collagens for broader applications than regenerative chondrocyte implantations (autologous chondrocyte implantation or characterized chondrocyte implantation) alone [56].

Under our conditions, COL2 mRNA abundances measured by quantitative PCR correlated well with protein synthesis as determined by Western blots (figures 2 and 3). The same observation holds for COL1 expression in the early passages, but not for COL1 expression in the later passages.

Hypertonicity induced an increase in NFAT5 abundance, and protein synthesis rates were found to be proportional to the increase in mRNA in MDCK cells [28] and mIMCD3 cells [27]. NFAT5 mRNA is expressed abundantly in chondrocytes throughout passages and is further induced by hypertonicity. However, we failed to show NFAT5 protein expression by Western blotting. Whether this failure is due to low protein abundance in our cells or technical issues such as poor extraction efficiency of this very large transcription factor remains to be elucidated in future experiments.

Hypertonicity induces cell shrinkage, which may activate Na+, K+, or 2Cl- co-transport, allowing cellular accumulation of NaCl and KCl. The beneficial effects on chondrogenic marker gene expression therefore could have been caused by accumulation of specific inorganic ions or specific channel activity rather than primarily tonicity-mediated effects. We used NMDG-Cl, a bulky substitute for small cations that is impermeable to almost all known channels [57], and sucrose to exclude sodium-specific or chloride-specific effects. We were not able to detect any significant differences in gene expression patterns between the NaCl, NMDG-Cl or sucrose methods of tonicity alteration (data not shown).

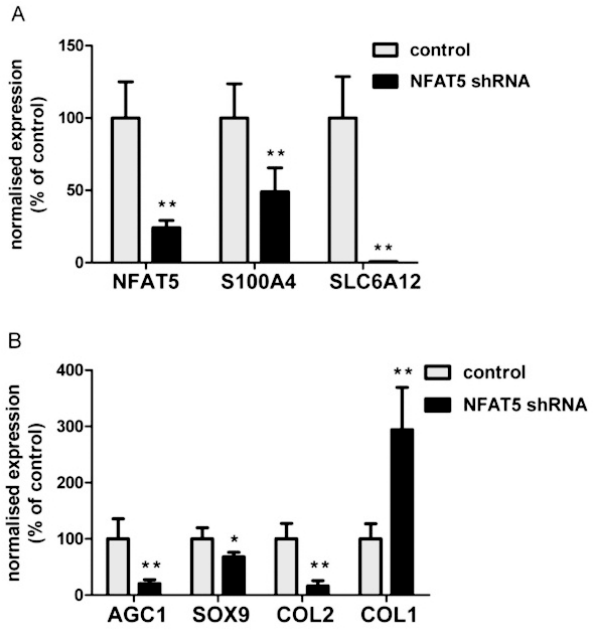

Figure 5: Nuclear factor of activated T-cells 5 knockdown inhibits hypertonicity-induced chondrogenic marker expression. (a) Relative expression of nuclear factor of activated T-cells 5 (NFAT5) and its target genes S100A4 and SLC6A12 in transduced chondrocytes either expressing (NFAT5 shRNA) or not expressing (control) NFAT5-specific shRNAs, 24 hours after increasing tonicity to 380 mOsm. (b) Effects of NFAT5 knockdown on chondrogenic markers AGC1, SOX9, COL2 and COL1. Data are mean ± standard deviation, n = 6. Differences from cells transduced with control virus are indicated: *P < 0.05 and **P < 0.01.

As our initial studies concerned adult HACs obtained from OA knee joints, we aimed at eliminating interpretation bias due to the pathological state of these cells. Using identically challenged NHACs, we showed that these chondrocytes react similarly to the same order of tonicity with respect to our marker genes: 380 mOsm significantly delayed the phenotypical deterioration of NHACs as observed in control medium. This may imply that physiological tonicity, postulated to be around 380 mOsm for chondrocytes, is sensed by OA cells and normal cells in a similar fashion. We observed a slightly faster decrease in AGC1 and COL2 mRNA levels in P2 and P3

NHACs as compared with OA HACs. Late-stage OA chondrocytes from fibrillated areas are dedifferentiated, flattened cells. The loss of a proper spherical shape as an integral part of the chondrocytes phenotype [58, 59] involves cytoskeletal changes [60]. Exposing these cells to physiological tonicity as a redifferentiation stimulus probably induces a more enduring response as compared with spherical, normal chondrocytes. Cell-based therapies using the latter are usually restricted to younger individuals after traumatic insults. Autologous chondrocyte implantation employing OA cells may benefit relatively more from a hypertonic treatment protocol.

The precise molecular mechanism by which tonicity is sensed by cells is still poorly understood. Hypertonicity increased NFAT5 mRNA abundances have been shown for other cell types [26-28]. NFAT5 is thus accepted as key transcription factor participating in the mammalian hypertonic stress response. Our study is the first showing the functional expression of NFAT5 in HACs. In both OA and normal chondrocytes, cellular NFAT5 mRNA levels are increased by 380 mOsm. In addition, mRNA levels of the generally accepted NFAT5 target genes, S100A4 and SLC6A12 [20, 61], were induced accordingly after hypertonic challenge, underscoring an involvement of NFAT5. It has recently been suggested that guanine nucleotide exchange factors near the plasma membrane may be activated through cytoskeleton changes or by changes in interactions with putative osmosensors at the cell membrane in other cells [62]. The sensation of such basic responses might not be different in chondrocytes than in other cells. Rho-type small G proteins [63] and p38 kinases [64, 65] might also act upstream of NFAT5 in chondrocytes. In IMCD cells, p38 mitogen-activated protein kinase (MAPK) signaling was recently also shown to be involved in the NFAT5-mediated hypertonic induction of the osmosensitive [66, 67] serine-threonine protein kinase Sgk-1 [68, 69]. As p38 MAPK plays important roles in chondrocytes and seems to be necessary for NFAT5 expression [20], further experiments employing pharmacological inhibition or knockdown experiments in HACs will hopefully shed more light into this signaling cascade in chondrocytes.

An increase in NFAT5 mRNA is usually transient with a cell type-dependent time course and a twofold to fourfold upregulation [26, 28], which fits

with our data. NFAT5 mRNA abundance might rapidly increase upon hypertonic stress by a transient increase in its mRNA stability, mediated by its 5'-untranslated region [27]. Whether 380 mOsm is a sufficiently high tonicity to explain our increase in mRNA by this phenomenon, or whether active transcription is involved, has to be addressed in other studies. Interestingly, Tew and colleagues showed very recently that the mRNA of SOX9, an important regulator of COL2 expression, is stabilized by supraphysiological tonicity [70]. Therefore, 380 mOsm might also directly contribute to SOX9 mRNA stability and abundance in our experiment, rather than elevating promoter activity. COL2 regulation could thus be an indirect effect of tonicity.

Interestingly, AGC1 seems to be more stably expressed in cultures maintained at 280 mOsm compared with 380 mOsm, with a lower overall expression in the former condition. Effects of tonicity on promoter activity and mRNA stability of AGC1 are incompletely understood. Other groups have described the complexity of osmotic stress on gene expression [71, 72]. It is tempting to speculate that gene expression may be influenced by morphological changes between our conditions: while cells cultured at 380 mOsm are rather round, cells cultured in monolayer at 280 mOsm are rather flat and more fibroblast-like (see figure 1). Although we did not investigate actin stress fiber formation in the present study, they are usually more pronounced in fibroblastic cells and have been shown to suppress SOX9 mRNA levels in chondrocytes [50].

Aggrecan expression, however, has been reported to be influenced by both hypertonicity and hypotonicity [4, 8]. The promoter regions of both collagen type II and AGC1 contain a plethora of potential other binding sites for transcriptional enhancers and suppressors, such as SOX5/6 [73, 74], Barx2 [75], β-catenin [76], c-Maf [77], PIAS [78], TRAP230 [79], Bapx1 [80], and C/EBP and NF-κB [81]. Chondrogenic differentiation and the SOX9 dependency of aggrecan and collagen expression may also be differentially modulated by these transcriptional cofactors under different tonicities. Interestingly, while the SOX9 dependency of COL2A1 expression has been unequivocally shown, it may not actually be a key regulator of COL2A1 promoter activity in human adult articular chondrocytes [82]. Of

note, the human aggrecan promoter sequence has been shown to contain a conserved NFAT5 binding site [83]. In nucleus pulposus cells, SOX9-mediated aggrecan expression has recently been shown to critically depend on PI3K/AKT signaling [84]. Moreover, while high NaCl rapidly activates p38 MAPK, its action can be isoform specific and may exert opposing effects on NFAT5 [85], which in turn may influence COL2A1 and AGC1 transcription differently in a tonicity dependent manner. We are therefore currently looking into the underlying molecular mechanisms regulating AGC1 and COL2 expression in both conditions.

With respect to regenerative medical applications, the high-end hypertonic conditions used by Tew and colleagues can be considered a limitation of that study. In our hands, these tonicity levels (≥ 480 mOsm) induced chondrocyte death within 48 hours (figure 1D) and are probably not applicable for chondrocyte expansion culture. To ensure sufficient cell numbers for cell-based repair techniques, the proliferation capacity of the isolated chondrocytes should not be compromised. Cell numbers generally need to be increased during two passages (>4 to 10 times) for clinical application [86, 87]. We found that supraphysiological conditions (480 mOsm and 580 mOsm) clearly compromised survival rates, which is in agreement with data by Racz and colleagues [17]. From our data, we conclude that about 380 mOsm is optimal for both isolation and in vitro expansion culture of HACs.

NFAT5 knockdown downregulates its own transcription by 75% and compromises target gene induction (figure 5), being in line with functionally active NFAT5 in chondrocytes. Constitutive homodimeric NFAT5 molecules encircle DNA rather independently of tonicity in solution [88], enabling NFAT5 to exert its biological activity over a wide tonicity range [89, 90]. It is thus reasonable to assume that NFAT5 activity is not generally compromised at 380 mOsm. However, other aspects are involved in the regulation of NFAT5 as well as its target genes. Like other proteins larger than 50 kDa [91], NFAT5 depends on nuclear localization and export sequences for its nuclear translocation [26, 88, 91]. In most cells, NFAT5 is equally distributed between the cytoplasm and the nucleus at physiological tonicity (±300 mOsm), whereas at 500 mOsm most of it

localizes to the nucleus [19, 26, 89].

To demonstrate that the hypertonicity-induced chondrogenic marker expression was indeed mediated by NFAT5, we used RNAi to confirm that knockdown of NFAT5 significantly inhibited hypertonic induction of its own transcription as discussed before, significantly suppressed the tonicity-mediated induction of known NFAT5 targets, and, most importantly, significantly eliminated the hypertonicity-mediated mRNA expression of chondrogenic marker genes (COL2, AGC1, SOX9 and COL1).

Conclusions

We have shown that isolation and expansion of adult HACs in culture medium of physiological tonicity (380 mOsm) improves chondrogenic marker expression and extracellular matrix production through NFAT5. We identified NFAT5 as a novel molecular target preserving chondrocytic marker expression. Our data provide valuable insights for the development of strategies for cellbased repair of chondral lesions, and contribute to the understanding of mechanisms involving OA.

References

1. Basic Orthopaedic Biomechanics, ed. V.C. Mow, Hayes W.C. 1997, Philadelphia, PA: Lippincott-Raven.
2. Lesperance, L.M., M.L. Gray, and D. Burstein, Determination of fixed charge density in cartilage using nuclear magnetic resonance. J Orthop Res, 1992. 10(1): p. 1-13.
3. Maroudas, A.I., Balance between swelling pressure and collagen tension in normal and degenerate cartilage. Nature, 1976. 260(5554): p. 808-9.
4. Urban, J.P., A.C. Hall, and K.A. Gehl, Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. J Cell Physiol, 1993. 154(2): p. 262-70.
5. Urban, J.P., The chondrocyte: a cell under pressure. Br J Rheumatol, 1994. 33(10): p. 901-8.
6. Bank, R.A., et al., The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. Arthritis Rheum, 2000. 43(10): p. 2202-10.
7. Bush, P.G. and A.C. Hall, Passive osmotic properties of in situ human articular chondrocytes within non-degenerate and degenerate cartilage. J Cell Physiol, 2005. 204(1): p. 309-19.
8. Palmer, G.D., et al., Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading. Osteoarthritis Cartilage, 2001. 9(8): p. 761-70.
9. Bush, P.G. and A.C. Hall, Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. J Cell Physiol, 2001. 187(3): p. 304-14.
10. Hung, C.T., et al., Disparate aggrecan gene expression in chondrocytes subjected to hypotonic and hypertonic loading in 2D and 3D culture. Biorheology, 2003. 40(1-3): p. 61-72.
11. Negoro, K., et al., Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three-dimensional culture system. Clin Exp Rheumatol, 2008. 26(4): p. 534-41.
12. Burg, M.B., Macromolecular crowding as a cell volume sensor. Cell Physiol Biochem, 2000. 10(5-6): p. 251-6.
13. Neuhofer, W., et al., Regulation of TonEBP transcriptional activator in MDCK cells following changes in ambient tonicity. Am J Physiol Cell

Physiol, 2002. 283(6): p. C1604-11.
14. Yancey, P.H., et al., Living with water stress: evolution of osmolyte systems. Science, 1982. 217(4566): p. 1214-22.
15. Kultz, D. and D. Chakravarty, Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. Proc Natl Acad Sci U S A, 2001. 98(4): p. 1999-2004.
16. Woo, S.K., S.D. Lee, and H.M. Kwon, TonEBP transcriptional activator in the cellular response to increased osmolality. Pflugers Arch, 2002. 444(5): p. 579-85.
17. Racz, B., et al., Hyperosmotic stress-induced apoptotic signaling pathways in chondrocytes. Bone, 2007. 40(6): p. 1536-43.
18. Burg, M.B., E.D. Kwon, and D. Kultz, Regulation of gene expression by hypertonicity. Annu Rev Physiol, 1997. 59: p. 437-55.
19. Miyakawa, H., et al., Tonicity-responsive enhancer binding protein, a rel-like protein that stimulates transcription in response to hypertonicity. Proc Natl Acad Sci U S A, 1999. 96(5): p. 2538-42.
20. Lopez-Rodriguez, C., et al., Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression. Proc Natl Acad Sci U S A, 2004. 101(8): p. 2392-7.
21. Na, K.Y., et al., Silencing of TonEBP/NFAT5 transcriptional activator by RNA interference. J Am Soc Nephrol, 2003. 14(2): p. 283-8.
22. Kasono, K., et al., Hypertonicity regulates the aquaporin-2 promoter independently of arginine vasopressin. Nephrol Dial Transplant, 2005. 20(3): p. 509-15.
23. Chen, M., et al., Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metastasin. J Biol Chem, 2009. 284(3): p. 1484-94.
24. Ito, T., et al., Taurine depletion caused by knocking out the taurine transporter gene leads to cardiomyopathy with cardiac atrophy. J Mol Cell Cardiol, 2008. 44(5): p. 927-37.
25. Nakamura, N. and K. Takenaga, Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. Clin Exp Metastasis, 1998. 16(5): p. 471-9.
26. Ko, B.C., et al., Purification, identification, and characterization of an osmotic response element binding protein. Biochem Biophys Res Commun, 2000. 270(1): p. 52-61.

27. Cai, Q., J.D. Ferraris, and M.B. Burg, High NaCl increases TonEBP/OREBP mRNA and protein by stabilizing its mRNA. Am J Physiol Renal Physiol, 2005. 289(4): p. F803-7.
28. Woo, S.K., et al., Bidirectional regulation of tonicity-responsive enhancer binding protein in response to changes in tonicity. Am J Physiol Renal Physiol, 2000. 278(6): p. F1006-12.
29. Morancho, B., et al., Analysis of the transcriptional activity of endogenous NFAT5 in primary cells using transgenic NFAT-luciferase reporter mice. BMC Mol Biol, 2008. 9: p. 13.
30. Widuchowski, W., J. Widuchowski, and T. Trzaska, Articular cartilage defects: study of 25,124 knee arthroscopies. Knee, 2007. 14(3): p. 177-82.
31. Brittberg, M., et al., Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med, 1994. 331(14): p. 889-95.
32. Saris, D.B., et al., Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. Am J Sports Med, 2008. 36(2): p. 235-46.
33. von der Mark, K., et al., Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature, 1977. 267(5611): p. 531-2.
34. Schnabel, M., et al., Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage, 2002. 10(1): p. 62-70.
35. Benya, P.D., S.R. Padilla, and M.E. Nimni, Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell, 1978. 15(4): p. 1313-21.
36. Setton, L.A., et al., Altered structure-function relationships for articular cartilage in human osteoarthritis and an experimental canine model. Agents Actions Suppl, 1993. 39: p. 27-48.
37. Loeser, R.F., Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis Cartilage, 2009. 17(8): p. 971-9.
38. Gouttenoire, J., et al., Modulation of collagen synthesis in normal and osteoarthritic cartilage. Biorheology, 2004. 41(3-4): p. 535-42.
39. Das, R.H., et al., Effects of individual control of pH and hypoxia in

chondrocyte culture. J Orthop Res, 2010. 28(4): p. 537-45.
40. Karsten, U. and A. Wollenberger, Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. Anal Biochem, 1977. 77(2): p. 464-70.
41. de Mos, M., et al., Can platelet-rich plasma enhance tendon repair? A cell culture study. Am J Sports Med, 2008. 36(6): p. 1171-8.
42. Stewart, S.A., et al., Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA, 2003. 9(4): p. 493-501.
43. Sigma-Aldrich. Available from: http://www.sigmaaldrich.com.
44. Dull, T., et al., A third-generation lentivirus vector with a conditional packaging system. J Virol, 1998. 72(11): p. 8463-71.
45. Zufferey, R., et al., Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol, 1998. 72(12): p. 9873-80.
46. Schambach, A., et al., Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther, 2006. 13(7): p. 641-5.
47. Follenzi, A. and L. Naldini, HIV-based vectors. Preparation and use. Methods Mol Med, 2002. 69: p. 259-74.
48. Uitterlinden, E.J., et al., Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage, 2006. 14(3): p. 250-7.
49. van der Windt, A.E., et al., Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGFbeta1 production. Tissue Eng Part A, 2010. 16(1): p. 1-10.
50. Tew, S.R. and T.E. Hardingham, Regulation of SOX9 mRNA in human articular chondrocytes involving p38 MAPK activation and mRNA stabilization. J Biol Chem, 2006. 281(51): p. 39471-9.
51. Mandl, E.W., et al., Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol, 2004. 23(4): p. 231-41.
52. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001. 25(4): p. 402-8.
53. Kraveka, J.M., et al., Immunoprecipitation of human telomerase

reverse transcriptase with telomerase activity. Anal Biochem, 2001. 291(1): p. 166-9.
54. ImageJ 1.42 software.
55. Benjamin, M. and J.R. Ralphs, Biology of fibrocartilage cells. Int Rev Cytol, 2004. 233: p. 1-45.
56. Wuertz, K., et al., Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells. J Orthop Res, 2007. 25(11): p. 1513-22.
57. Duranton, C., et al., Organic osmolyte permeabilities of the malaria-induced anion conductances in human erythrocytes. J Gen Physiol, 2004. 123(4): p. 417-26.
58. Darling, E.M. and K.A. Athanasiou, Retaining zonal chondrocyte phenotype by means of novel growth environments. Tissue Eng, 2005. 11(3-4): p. 395-403.
59. Murphy, C.L. and A. Sambanis, Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes. Tissue Eng, 2001. 7(6): p. 791-803.
60. Darling, E.M., et al., Mechanical properties and gene expression of chondrocytes on micropatterned substrates following dedifferentiation in monolayer. Cell Mol Bioeng, 2009. 2(3): p. 395-404.
61. Jeon, U.S., et al., How tonicity regulates genes: story of TonEBP transcriptional activator. Acta Physiol (Oxf), 2006. 187(1-2): p. 241-7.
62. Kino, T., et al., Brx mediates the response of lymphocytes to osmotic stress through the activation of NFAT5. Sci Signal, 2009. 2(57): p. ra5.
63. Kelkar, N., C.L. Standen, and R.J. Davis, Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways. Mol Cell Biol, 2005. 25(7): p. 2733-43.
64. Lopez-Rodriguez, C., et al., NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. Proc Natl Acad Sci U S A, 1999. 96(13): p. 7214-9.
65. Ho, S.N., Intracellular water homeostasis and the mammalian cellular osmotic stress response. J Cell Physiol, 2006. 206(1): p. 9-15.
66. Nishida, Y., et al., Alteration of serum/glucocorticoid regulated kinase-1 (sgk-1) gene expression in rat hippocampus after transient global ischemia. Brain Res Mol Brain Res, 2004. 123(1-2): p. 121-5.
67. Rozansky, D.J., et al., Hypotonic induction of SGK1 and Na+ transport

in A6 cells. Am J Physiol Renal Physiol, 2002. 283(1): p. F105-13.
68. Chen, S., et al., Tonicity-dependent induction of Sgk1 expression has a potential role in dehydration-induced natriuresis in rodents. J Clin Invest, 2009. 119(6): p. 1647-58.
69. Webster, M.K., et al., Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Mol Cell Biol, 1993. 13(4): p. 2031-40.
70. Tew, S.R., et al., Hyperosmolarity regulates SOX9 mRNA posttranscriptionally in human articular chondrocytes. Am J Physiol Cell Physiol, 2009. 297(4): p. C898-906.
71. Chao, P.H., A.C. West, and C.T. Hung, Chondrocyte intracellular calcium, cytoskeletal organization, and gene expression responses to dynamic osmotic loading. Am J Physiol Cell Physiol, 2006. 291(4): p. C718-25.
72. Finan, J.D. and F. Guilak, The effects of osmotic stress on the structure and function of the cell nucleus. J Cell Biochem, 2010. 109(3): p. 460-7.
73. Ikeda, T., et al., The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum, 2004. 50(11): p. 3561-73.
74. Lefebvre, V., R.R. Behringer, and B. de Crombrugghe, L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage, 2001. 9 Suppl A: p. S69-75.
75. Meech, R., et al., The homeobox transcription factor Barx2 regulates chondrogenesis during limb development. Development, 2005. 132(9): p. 2135-46.
76. Akiyama, H., et al., Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev, 2004. 18(9): p. 1072-87.
77. Huang, W., et al., A new long form of c-Maf cooperates with Sox9 to activate the type II collagen gene. J Biol Chem, 2002. 277(52): p. 50668-75.
78. Hattori, T., et al., Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9. J Biol Chem, 2006. 281(20): p. 14417-28.
79. Zhou, R., et al., SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic Acids Res, 2002.

30(14): p. 3245-52.
80. Yamashita, S., et al., Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res, 2009. 315(13): p. 2231-40.
81. Ushita, M., et al., Transcriptional induction of SOX9 by NF-kappaB family member RelA in chondrogenic cells. Osteoarthritis Cartilage, 2009. 17(8): p. 1065-75.
82. Aigner, T., et al., SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol, 2003. 22(4): p. 363-72.
83. Tsai, T.T., et al., TonEBP/OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc. J Biol Chem, 2006. 281(35): p. 25416-24.
84. Cheng, C.C., et al., PI3K/AKT regulates aggrecan gene expression by modulating Sox9 expression and activity in nucleus pulposus cells of the intervertebral disc. J Cell Physiol, 2009. 221(3): p. 668-76.
85. Zhou, X., et al., MKP-1 inhibits high NaCl-induced activation of p38 but does not inhibit the activation of TonEBP/OREBP: opposite roles of p38alpha and p38delta. Proc Natl Acad Sci U S A, 2008. 105(14): p. 5620-5.
86. Brittberg, M., Autologous chondrocyte implantation--technique and long-term follow-up. Injury, 2008. 39 Suppl 1: p. S40-9.
87. Brittberg, M., et al., Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. J Bone Joint Surg Am, 2003. 85-A Suppl 3: p. 109-15.
88. Lopez-Rodriguez, C., et al., NF-AT5: the NF-AT family of transcription factors expands in a new direction. Cold Spring Harb Symp Quant Biol, 1999. 64: p. 517-26.
89. Lopez-Rodriguez, C., et al., Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. Immunity, 2001. 15(1): p. 47-58.
90. Lee, S.D., S.K. Woo, and H.M. Kwon, Dimerization is required for phosphorylation and DNA binding of TonEBP/NFAT5. Biochem Biophys Res Commun, 2002. 294(5): p. 968-75.
91. Cyert, M.S., Regulation of nuclear localization during signaling. J Biol Chem, 2001. 276(24): p. 20805-8.

6

Chapter 6

The role of hypoxia in bone marrow–derived mesenchymal stem cells: considerations for regenerative medicine approaches

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Published as Das RHJ et al., The Role of Hypoxia in Bone Marrow–Derived Mesenchymal Stem Cells: Considerations for Regenerative Medicine Approaches, Tissue Eng Part B (2009)
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Summary

Bone marrow–derived mesenchymal stem cells (MSCs) have demonstrated potential for regenerative medicine strategies. Knowledge of the way these cells respond to their environment in in-vitro culture and after implantation in vivo is crucial for successful therapy. Oxygen tension plays a pivotal role in both situations. In vivo, a hypoxic environment can lead to apoptosis, but hypoxic preconditioning of MSCs and overexpression of prosurvival genes like Akt can reduce hypoxia-induced cell death. In cell culture, hypoxia can increase proliferation rates and enhance differentiation along the different mesenchymal lineages. Hypoxia also modulates the paracrine activity of MSCs, causing upregulation of various secretable factors, among which are important angiogenic factors such as vascular endothelial growth factor and interleukin-6 (IL6). Finally, hypoxia plays an important role in mobilization and homing of MSCs, primarily by its ability to induce stromal cell–derived factor-1 expression along with its receptor CXCR4. This article reviews the current literature on the effects of hypoxia on MSCs and aims to elucidate its potential role in regenerative medicine strategies.

Introduction

The use of bone marrow–derived stromal cells in tissue culture was first described by Friedenstein as early as 1966.[1-5] Since then, these cells have been termed "mesenchymal stem cells" (MSCs) by Caplan et al.[6] for their potential to differentiate along multiple mesenchymal lineages. Both terms are now in common use. The use of MSCs for tissue engineering purposes and their potential to differentiate along the bone, cartilage, fat, and muscle lineages have been demonstrated, and their usefulness for regenerative medicine approaches is clear.[7-18] MSCs might exert their beneficial effects in various ways. They might differentiate directly and function as a new cell type, but they might also function indirectly through the release of trophic factors that fulfill an endocrine/paracrine role. Their harvest from autologous donor sites is relatively harmless and they can readily be cultured in vitro to obtain sufficient cell numbers for reimplantation.

The issue of optimal culture conditions for cells has been under investigation for many years. An important consideration is the optimum oxygen tension in which to culture cells. Physiological oxygen tension varies from as much as 12% in the blood to as low as 1% in the deep zone of cartilage regions.[19] The oxygen tension in bone marrow has been described to be between 4% and 7%[20] or perhaps as low as 1–2%.[21, 22] In any case, the oxygen tension is considerably lower in vivo than the atmospheric oxygen tension (21%) of standard cell culture.[23]

Culture in oxygen tension that more closely represents the in-vivo situation can have effects in several ways. Cooper et al.[24] and Zwartouw and Westwood[25] described as early as 1958 that some cells proliferate more rapidly in oxygen tensions that are lower than atmospheric. In their article, Lennon et al.[20] also describe several other studies where many different cell types showed increased proliferative rates when cultured under lower oxygen tensions. In recent years, interest in the potential of MSCs for regenerative medicine applications and the availability of more convenient methods to alter oxygen tension in culture have led to publication of many articles assessing the effects of low oxygen tension on MSC behavior

and function. The issues of tissue ischemia, for example, after cardiac arrest, and tissue-engineered construct failure due to lack of oxygen are receiving growing interest in the field of regenerative medicine.[26-32]

Complementary to a recent review by Ma et al.,[22] this review focuses on the role of hypoxia in critical aspects of bone marrow–derived MSC behavior relevant to researchers in the fields of tissue engineering and regenerative medicine (including survival, proliferation, differentiation, and overall behavior) and addresses underlying mechanisms. This review gives a comprehensive overview of studies performed to assess the effects of low oxygen (pO2<10% for this review) on MSCs. This knowledge may help to better tailor culture conditions to achieve the desired outcome of a regenerative medicine approach.

Survival

An important possible clinical application of MSCs is their use in the repair of cardiac tissue after myocardial infarction. Unfortunately, 99% of MSCs die within 3–4 days after transplantation into the ischemic heart.[33-35] The ability to survive in an environment that is deprived of both oxygen and nutrient supply is necessary for successful myocardial tissue repair, as prolonged viability is a prerequisite for angiogenesis.[35, 36] Similarly, tissue engineering of cell-seeded constructs above a critical size (approximately 4–5mm2) is often problematic because availability of oxygen and nutrients for cells is limited by diffusion in these avascular constructs. This means that cells residing deeper in the construct experience a hypoxic and nutrient-deficient environment. Although cell death appears to be less severe in other applications (e.g., in intervertebral disk[37, 38] or cartilage repair[39]), for regenerative medicine approaches it is absolutely critical that cells survive long enough to elicit a repair response.

Under ischemic conditions, cells cannot rely on oxidative phosphorylation for the generation of adenosine triphosphate (ATP), because this pathway depends on the availability of oxygen. The glycolytic pathway, which functions independently of oxygen, provides a means for cells to generate energy under conditions where oxygen availability is limited. The ability to

successfully switch metabolic pathways from aerobic to anaerobic and the availability of abundant glucose for ATP production are key elements for cell survival under hypoxic conditions. This is illustrated in studies by Zhu et al.[40] and Potier et al.,[41] who showed that rat MSCs were able to withstand hypoxia only when they were not also deprived of serum. Indeed, MSCs seem to employ the glycolytic pathway to maintain viability when faced with hypoxic challenges.[42] Consequently, it was concluded that serum deprivation, not hypoxia, is the main reason for apoptotic cell death of MSCs in ischemia. However, hypoxia also leads to apoptotic cell death after prolonged exposure. Mitochondrial dysfunction leading to increased Caspase-3 activation is believed to be a major contributor in this process.[40]

Contrary to other cell types like cardiomyocytes (which fail to survive hypoxia even for 4 h), MSCs can withstand hypoxia ($pO2<1\%$) for at least 48 h.[43, 44] Unlike cardiomyocytes, MSCs are able to derive the bulk of their ATP from glycolysis; moreover, the ATP requirement for MSC survival is low.[43] In this scenario, the buildup of lactate due to increased glycolysis and subsequent drop in pH might become an inhibiting factor in the long term.

Cells can respond to oxygen tension through transcription factors, most notably hypoxia inducible factor-1a (HIF-1a). HIF-1a is degraded under normoxic conditions, but stabilized under hypoxic conditions, because the prolyl-hydroxylase that primes HIF-1a for degradation uses oxygen as a cofactor.[45] Stabilization of HIF-1a also appears to depend on hypoxiainduced increase of phosphorylated Akt and p38 mitogen–activated protein kinase (p38MAPK).[46] Stabilized HIF-1a translocates to the nucleus, allowing it to dimerize with HIF-1b, so it can bind to promoter regions of hypoxia-responsive genes. One of the target genes of HIF-1 is the glucose-6-phosphate transporter, which controls the rate-limiting step in gluconeogenesis by glucose-6-phosphatase.[47] The increased availability of glucose (through upregulated gluconeogenesis) would help extend the survival of MSCs under hypoxic and serum-deprived conditions, and it is believed that this is one of the mechanisms by which MSCs survive a hypoxic insult.[47]

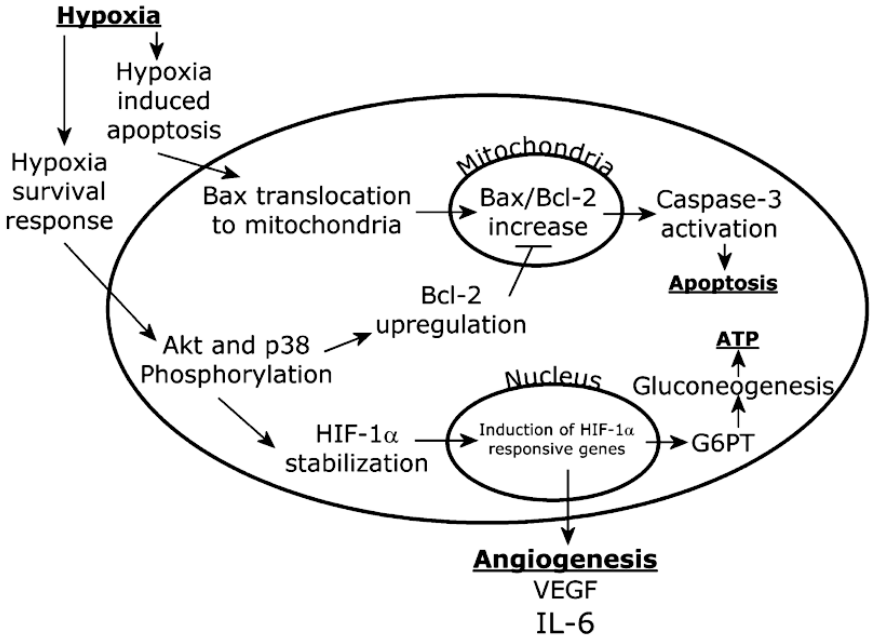

Figure 1: Mesenchymal stem cell response to hypoxia. Bax accumulates in the mitochondria, increasing the Bax/Bcl-2 ratio and causing mitochondrial dysfunction. Subsequent activation of Caspase-3 leads to apoptosis.[40] In a survival response, Akt and p38 phosphorylation leads to stabilization of hypoxia-inducible factor-1α (HIF-1α)[46] and upregulation of Bcl-2.[48] Bcl-2 upregulation can counteract the accumulation of Bax, leading to protection from apoptosis. HIF-1α stabilization and translocation leads to induction of HIF-1α target genes, among which are glucose-6-phosphatase transporter[47] and important angiogenic factors such as vascular endothelial growth factor (VEGF) and IL6.[51]

Although MSCs are able to withstand hypoxic conditions for up to a few days, they need to survive longer in ischemic tissue. Priming cultured MSCs by hypoxic reconditioning can enhance their survivability by HIF-1–dependent upregulation of erythropoietin (EPO)/erythropoietin receptor

(EPOR) and antiapoptotic factors Bcl-2 and bcl-xL and accompanied decreased caspase-3 activation.[48, 49] Additionally, proangiogenic factor production, for example, vascular endothelial growth factor (VEGF) and (IL6), is stimulated by hypoxic preconditioning. [48] These beneficial effects are regulated by a complex array of signaling pathways that include the Akt and ERK pathways.[50-52] Akt, activated by hypoxia and other stimuli, has been shown to be necessary for cell survival,[53] likely by its glucose metabolism–modulating effects. Transplantation of genetically engineered rat MSCs, which retrovirally expressed the prosurvival gene Akt1, into ischemic rat myocardium showed reduced inflammation and cardiomyocyte hypertrophy, while restoring 80–90% of lost myocardial volume.[54] Hypoxia-induced Akt activation reduced proapoptotic Bax expression by 27% while increasing Bcl-2 expression by 50%. The overall reduction in apoptosis amounted to 80% within 24 h compared with green fluorescent protein (GFP) controls. Gnecchi et al.[55] went a step further and showed that in a left coronary artery ligation model (rat) conditioned medium from hypoxic Akt-transformed MSCs was sufficient to inhibit subsequent hypoxia-induced apoptosis. Figure 1 shows the interplay of major factors involved in MSC survival response to hypoxia and hypoxia-induced apoptosis.

Clearly, the poor long-term viability in vivo limits the therapeutic applications of MSCs, but with the uncovering of signaling and apoptotic pathways involved in MSC survival, dedicated strategies can be developed. In an attempt to prolong in-vivo survival, Xu et al.[56] found that Lovastatin could counteract hypoxia-induced apoptosis by upregulating pAkt and ERK1/2. Another protective effect was found for Lysophospotidic acid.[57] In a different approach, Liu et al.[58] adenovirally transfected MSCs with angiogenin and found that MSC survival was enhanced in vivo compared with untreated MSCs. Similarly, Li et al.[59] found increased survival with MSCs that were modified to overexpress the antiapoptotic Bcl-2 gene, while Tang et al.[30] used overexpression of the hypoxia-regulated heme-oxygenase-1 gene to enhance MSC survival. Unfortunately, genetic modification of MSCs has only limited clinical relevance because the process is very labor intensive and carries risks inherent to genetic modification.

Proliferation

Although physiological oxygen tensions even in healthy tissue are well below 21% pO2, more often than not cells are cultured at this oxygen tension. Culture under oxygen tension in the physiological range can affect the rate of proliferation of various cell types.[20] Indeed, Lennon et al. showed that culture of MSCs, derived from bone marrow of 6–12-week-old rats, under 5% pO2 resulted in roughly 40% higher cell number at first passage compared with culture under 21% pO2.[20] The results appeared to persist even after passaging. Grayson et al.[60] showed that human MSCs also displayed enhanced proliferation under reduced oxygen tension (2% pO2) for seven passages, resulting in a 30-fold increase in cell number compared with normoxic culture.[60]
On three-dimensional substrates, which are commonly used in tissue engineering applications, MSCs displayed a prolonged lag phase in early culture under reduced oxygen tension (2% pO2). However, MSCs cultured under 2% pO2 did not display a plateau-phase, but rather continued their proliferation even after 23 days of culture, unlike MSCs cultured under 21% pO2.[42]

From their studies, Grayson et al.[42] concluded that reduced oxygen did not cause faster proliferation of MSCs, but rather extended the duration of their proliferation. In contrast, D'Ippolito et al. concluded that reduced oxygen tension decreased population-doubling time in their marrow isolated adult multilineage inducible cells, with a maximum at 3% pO2.[61] This result is more in line with other studies that found faster proliferation of their bone marrow–derived mononuclear cells under 5% pO2[62] or MSCs under 8% pO2.[63] The latter showed that hypoxia increased the number of G2/S/M period cells. Martin-Rendon et al. also attributed increased proliferation, even short-term exposure to 1.5% pO2, to more rapid cell cycle progression,[64] but Rosova et al.[52] did not find this increase when evaluating the effects of hypoxia on the short term (16 h). Contrary to these findings, Hung et al.[65] found lower cell numbers when expanding their human MSCs at 1% pO2 in 17% fetal bovine serum, but the effect was smaller at higher seeding density.

Overall, reduced oxygen tension in the physiological range increased MSC number obtained after monolayer and three dimensional culture. Whether extended proliferation capacity or decreased population doubling time is responsible appears to depend on the exact pO2 as well as cell type and other culture conditions. The mechanisms underlying this altered proliferation are currently unknown, but Yun et al. suggested a role for estradiol-17β.[66] Table 1 summarizes the findings of the effects of hypoxia on proliferation.

Expansion of MSCs under hypoxic conditions does not affect their multilineage potential. Rather, it appeared to even increase expression of Oct-4.[60] Oct-4 is an established indicator of enhanced pluripotency[61] and might be regulated upstream by HIF-2a[60] or HIF-1a.[61]

Table 1
Effects of Hypoxia on proliferation

O_2 tension (%)	Cell type	Serum type/ concentration	Duration	Proliferation	Author	Ref.
1	Human MSCs	17% FBS	10 days	Decreased rate of proliferation	Hung	65
1-3	Human MSCs from BMA	Serum-free	18 h	No effect of hypoxia on cell viability	Rosova	52
1, 3, 5, and 10	MIAMI	2% FBS for expansion	3, 7, 10, and 15 days	Increase, highest at 3% pO_2	D'Ippolito	61
1 and 50	BMMC (Cambrex, East Rutherford, NJ)	N/A (MSCGM [Cambrex])	24 h	1.61-fold increase in viable cell number	Martin-Rendon	64
2	Human MSCs	10% FBS	Up to 6 weeks	30-fold increase	Grayson	60
2	Human MSCs	10% FBS	Up to 30 days	Increase after initial lag	Grayson	42
5	MNC from BMA	10% fetal calf serum (FCS) or platelet lysate	P1-4 to 90% confluency	Decreased time to reach confluency	Carrancio	62
5	Rat MSCs	10% FBS	First passage	Mean increase in cell number of 41.2%	Lennon	20
8	Murine MSCs	10% FBS	7-8 days	2.8-fold increase	Ren	63

MSCs, mesenchymal stem cells; FBS, fetal bovine serum

Differentiation

The multilineage potential of MSCs is one reason for their appeal in regenerative medicine. Differentiation along the various mesenchymal lineages could be either enhanced or diminished under hypoxic culture. Various groups have assessed multilineage potential either after hypoxic preculture before differentiation or differentiation during hypoxia. Both approaches are grouped in this section.

Osteogenic differentiation

It is difficult to state clearly the effects of hypoxia on osteogenic differentiation. In an elegantly designed experiment, Lennon et al.[20] cultured cells for several passages under 5% pO2 and then either maintained them in 5% or 20% pO2 or switched to the opposite condition. After this they assessed osteogenic differentiation capacity finding that 5% pO2for the duration of the culture gave the largest osteogenic response followed by cells cultured first in normoxia then 5% pO2. Switching from 5% pO2 to normoxia or culturing completely in normoxia produced less mineralization by comparison. Several other articles have since described the effects of hypoxia on osteogenesis. Salim et al. found no difference between 21% pO2 and 2% pO2 on differentiation potential but observed diminished osteoblastic differentiation at 0.02% pO2.[67] They also found that as little as 6 h of anoxia prevented osteoblastic differentiation in the long term, and verified their results using osteoblasts. Grayson et al. found an initial increase in mineralization after a preculture period in 2% pO2 but observed no difference when culture was continued up to 19 days.[42] As with Grayson et al., Martin-Rendon et al.[64] also assessed differentiation potential of cells after a hypoxic preculture and also observed no difference in osteoblastic differentiation of cells. Potier et al.[44] demonstrated very variable results with some osteogenic genes being downregulated, while others remained unchanged. However, they also stated that they were unable to induce mineralization in these cells normally. In contrast D'Ippolito et al.,[61] Hung et al.,[65] and Muller et al.[68] observed reduced osteogenic capacity of cells cultured under hypoxia or after hypoxia. As is often the case, there were large differences in all experimental procedures, perhaps most significantly whether differentiation was induced under

hypoxic conditions or after a hypoxic preculture.

Chondrogenic differentiation

Fewer articles have assessed the effects of hypoxia on chondrogenic differentiation of MSCs despite the obvious link with the in-vivo situation (chondrocytes reside in a very hypoxic environment). The general consensus is that either a preculture under hypoxic conditions or differentiation under hypoxia increases chondrogenic gene expression[46, 64, 69] and other chondrogenic markers,[43] or at least has no effect.[62] Scherer et al.[70] showed that while 5% pO2 did not prevent chondrogenic differentiation in the presence of chondrogenic medium, it alone was not sufficient to induce chondrogenic differentiation. Little research has been performed examining the signaling mechanisms involved in differentiation under hypoxic conditions. However, Kanichai et al.[46] and Robins et al.[69] have elucidated some of the mechanisms involved in chondrogenesis. Upregulation of Sox9, an important transcription factor involved in chondrogenesis, was shown to involve HIF-1a under hypoxia and this involved both the p38MAPK and Akt pathways. Hirao et al. showed that this effect, also mediated by p38MAPK, was independent of Sox9, though this was observed in the C3H10T1/2 mesenchymal cell line.[71]

Adipogenic differentiation

Regarding adipogenic differentiation, it appears that hypoxia is also beneficial for differentiation along this lineage. Most articles report either an increase[42, 43, 63, 72]or no effect of hypoxia on adipogenesis.[60, 62] Only one article reports a significant drop in adipogenic activity when MSCs were cultured continuously at 1% pO2 for up to 21 days.[65]

In general, little work has been performed on understanding the mechanisms behind the alterations in behavior of MSCs under hypoxic conditions with the exceptions of a few articles. [46, 69]In conclusion, effects of hypoxia on osteo-, chondro-, and adipogenic differentiation capacity appear to be beneficial more often than nonexistent or negative. However, the exact outcome seems to depend heavily on the exact oxygen tension, time in culture, and the use of hypoxic preculture.

It should be noted that, as presented in Table II, the results discussed apply to cells derived from several different sources. For example, in this section

Table 2
Effects of Hypoxia on Differentiation

Oxygen tension (%)	Cell type	Differentiation during or post hypoxia	Osteogenesis	Chondrogenesis	Adipogenesis	Other	Author	References
0.2 and 2	Human MSC	Post	No effect 2% Decreased effect 0.2%	N/A	N/A	N/A	Salim	67
0 and 50	Rat MSC	Post	N/A	Normal	Normal	Cardiomyogenesis	Mylotte	43
1 or less	Human MSC	Post	Decreased	N/A	N/A	N/A	Potier	44
1	Human MSC	During/post	Decreased (during)/normal (post)	N/A	Decreased (during/normal (post)	N/A	Hung	51
1	Mouse ST2 stromal	During	N/A	Induced	N/A	N/A	Robins	69
1, 3, 5, and 10	MIAMI	During	Blocked at 3%	N/A	N/A	N/A	D'Ippolito	61
1 and 50	BMMC (Cambrex)	Post	Unaltered	Increased	Unaltered	N/A	Martin-Rendon	64
2	Human MSC	Post	Normal	N/A	Normal	N/A	Grayson	42
2	Rat MSC	During	N/A	Increased	N/A	N/A	Kanichai	46
5	MNC from BMA	Post	Normal	Normal	Normal	N/A	Carrancio	62
5	Rat MSC	During	Increased	N/A	N/A	N/A	Lennon	20

we discuss the results of the effects of hypoxia on the differentiation of immortalized human MSCs,[72] normal human MSCs,[42, 44, 60-62, 64, 67]rat MSCs,[20, 43, 46, 73] and mouse MSCs.[63, 69, 74]

Endocrine/Paracrine role

Apart from their own capacity to differentiate into various lineages, MSCs can play an important role in various processes via secretion of trophic factors. This has been evidenced, among others, by their ability to promote expansion and differentiation of hematopoietic stem cells,[75] enhance wound healing,[76] and support myocardial[77] and neuronal repair.[78] More evidence for trophic factor–mediated effects of MSCs can be found in their immunomodulatory role by inhibiting the proliferation of antigen-activated peripheral blood mononuclear cells and production of proinflammatory cytokines.[79-81] Inhibitory effects on various T cell subsets, B cells, natural killer cells, and antigen-presenting cells have been reported.[82] This can be achieved both through cell– membrane contact and through soluble factors.[83, 84]

Hypoxia can influence a broad spectrum of factors, as was revealed by screening with microarrays. Ohnishi et al. described that many genes were upregulated when rat MSCs were cultured for 24 h in 1% pO2 compared with normoxia.[85] Focusing on genes encoding secretory proteins, they describe upregulation of several molecules involved in cell proliferation and survival, such as VEGF-D, placental growth factor, pre-D-cell colony–enhancing factor 1, heparin-binding epidermal growth factor, and MMP9. For some of the genes they report on the effect of different oxygen tensions and time period of hypoxia. Most genes were upregulated even at 10% oxygen, with the effects becoming apparent after 24 h of hypoxia. The observation that most genes are regulated after 24 h was confirmed by Wu et al., who performed microarrays on a time course (4, 12, 24, 36, 48, and 72 h) using human MSCs at a pO2 of 3% and demonstrated that different genes are regulated via different time courses.[86] Although most genes were regulated after 24 h, as little as 4 h of hypoxia was reported to increase VEGF secretion and MT1 (MMP) expression and decrease MMP2. [74] Also, conditioned medium of MSCs after 6 h of hypoxia appeared to

give a larger increase in hypertrophy of cardiomyocytes than shorter or longer periods of hypoxia.[32]

Increased mRNA expression did not always result in increased secretion of factors in the medium. Human MSCs cultured for 72 h in 1% pO2 increased mRNA expression of fibroblast growth factor 2 (FGF2) and 7, IL1 and 6, placental growth factor, transforming growth factor-b, tumor necrosis factor (TNF-a), and VEGF, but only for VEGF, FGF2 and IL6 did this result in increased protein release in the medium.[87] The results of Muir et al. confirmed increased expression of VEGF by hypoxia, but the hs27 cell line derived from bone marrow expressed very low levels of FGF and HB-EGF.[88]

Potier et al.[44] cultured human MSCs with a slowly decreasing oxygen tension level. After 48 h of culture <1% oxygen was present and mRNA expression of transforming growth factor-β3 was decreased, whereas FGF2 and VEGF were increased. On the protein level VEGF was also increased, but for FGF2 no effect was found. In this study the secretion of IL6, IL8, and MCP1 was not significantly affected. In another study where human MSCs were cultured for 2 days in hypoxia in a serum-free medium (Potier used 10% FCS), IL6 and MCP1, next to VEGF, were found in increased amounts in the medium.[51]

Hypoxia not only affects the secretion of soluble factors but also upregulates CX3XR1 and CXCR4,[65, 89] as well as cMet (receptor for hepatocyte growth factor).[52] These receptors can increase MSC migration and homing to damaged areas. Although the immunomodulatory properties of MSCs are known to be promoted under inflammatory conditions (e.g., in response to interferon-γ (IFN-γ[90])), no studies were available for the effect of hypoxia on immunoregulatory properties of MSCs. Hypoxia appeared to regulate the increased production of soluble factors (at least of VEGF, FGF2, hepatocyte growth factor, and insulin-like growth factor (IGF)), similar to lipopolysaccharide or TNF-a, via an NF-kappaB–dependent mechanism,[91] which suggests that hypoxia might indeed regulate immunoregulatory properties too.

Thus hypoxia has been shown to influence the secretion of trophic factors as well as the membrane markers associated with migration and homing of MSCs. This suggests that hypoxic preconditioning of MSCs can be used to improve the performance of MSCs in regenerative medicine applications. Evidence for beneficial effects of hypoxic preconditioning of MSCs is provided by small animal experiments. Preconditioning MSCs for 24 h in hypoxia before implantation into myocardial infarction models resulted in less death and a better angiogenic potential of the MSCs.[48, 92] The optimal time period of hypoxia to obtain this effect is not known. Wang et al. used very short periods of hypoxia (10, 20, and 30 min) and found effects on viability and angiogenic properties already after 10 min, with larger effects after longer time periods of hypoxia.[93]

When MSCs were applied systemically, preconditioning demonstrated improved migration of MSCs to injured brain.[89] The authors demonstrated the role of increased CXCR4 by hypoxic MSCs that binds to stromal cell–derived factor-1a (SDF-1a) that is expressed in the damaged brain.

Recruitment and homing

The precise mechanism how MSCs are mobilized into the bloodstream is still not fully understood. Rochefort et al.[94] housed adult male rats 3 weeks in a hypoxic chamber, which caused chronic hypoxia[95] and compared this to animals housed at normoxia. This in vivo rat model for hypoxic MSC mobilization increased the pool of circulating MSCs 15-fold, while maintaining transdifferentiation potential and immunophenotype similar to that of MSCs. Moreover, this stimulus seemed to be relatively cell-type specific as the pool of hematopoietic precursors remained largely unaltered. However, chronic hypoxia could induce MSC mobilization by a number of direct and indirect mechanisms, including modification of local factors involved in the maintenance of MSCs. These factors can include altered peripheral blood concentrations of chemotactic factors specific for MSCs and/or increased vascular permeability favored by the neoangiogenesis (described in a model similar to that by Rochefort et al.).[96] Hypoxia also increases the in vitro migration capacity of MSCs, which is likely an MMP-dependent phenomenon.[74] Endothelial cells

under hypoxic conditions have been shown to stimulate MMP-2 and VEGF expression by MSCs.[97, 98] More recently, Rosova et al. found a possible role for hepatocyte growth factor and its receptor cMet, which is upregulated under hypoxia.[52] The ability of implanted MSCs to migrate to the site of damaged tissue has been shown in bone fractures and cartilage defects,[39] myocardial infarction,[99] and cerebral injury.[100] The question remains how MSCs are directed to the site of repair in vivo. This process of migration may involve various chemokines, cytokines, and integrins. Among the chemokines, the SDF-1/CXCR4 axis has been reported to be constitutively expressed in a wide range of tissues.[101] SDF-1 is a potent factor in mobilizing progenitors from the bone marrow niche.[64] SDF-1(CXCL12) and its receptor CXCR4 are crucial for homing and migration of stem cells, and the interaction of locally produced SDF-1 and CXCR4 expressed on the MSC surface plays an important role in the migration of transplanted cells. Although SDF-1 mediates homing of stem cells to bone marrow by binding to CXCR4 on circulating cells, the regulation of SDF-1 and its physiological role in peripheral tissue repair remain poorly understood. An elegant model of graded soft tissue ischemia in mice[102] showed that SDF-1 gene expression is regulated by HIF-1 in endothelial cells. A selective in vivo expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tensions was found. The recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1–induced expression of SDF-1. A middle cerebral artery occlusion model for brain ischemia was used by Wang et al.[89] to show that in MSCs expression of CXCR4 is also hypoxia-inducible. The authors demonstrated that migration of green fluorescent protein (GFP)-labeled MSCs to ischemic brain regions could be blocked by AMD3100, a CXCR4-specific antagonist. SDF-1 mRNA peaks after 3–5 days in ischemic cardiomyocytes.[103] Interestingly, the surface expression of CXCR4 diminishes with serial passages in vitro,[104, 105] while the intracellular majority of this receptor may be mobilized to the cell surface upon hypoxic stimulation. Targeting the expression of these two molecules might be a promising route to facilitate the therapeutic effects of MSCs in regenerative medicine.

Discussion

As with other cell types, there is no doubt that there are definite effects of hypoxia on MSC behavior.[19] Unlike some cell types, MSCs can survive hypoxic environments for several days by upregulating survival pathways and increasing glycolytic metabolism. Cell numbers are also increased when proliferating under low oxygen tension. Additionally, differentiation into different mesenchymal lineages can be enhanced by culture under some hypoxic conditions, but effects seem to depend on various conditions like exact oxygen tension, time in culture, and the use of hypoxic preculture. Finally, hypoxic conditions enhance the paracrine role of MSCs by altered trophic factor release and it also plays an import role in mobilizing MSCs and recruiting them to sites of injury. Clearly, hypoxia plays a crucial role in many aspects of MSC culture and therapy, but lack of consensus remains, especially when hypoxia is considered for differentiation. There are many considerations that can lead to this lack of consensus on findings. For example, the obvious consideration is choice of oxygen tension. Many researchers use atmospheric oxygen as the control but that is where the similarity ends. Fink et al. used a range of tensions (1%, 2%, 3%, 4%, and 6%) as did D'Ippolito et al. (1%, 3%, 5%, 10%, and 21%). Most other researchers use a single, defined oxygen tension for the duration of their study.[20, 42, 43, 46, 60, 62-67, 69, 73] Interestingly, some studies also used oxygen chelation methods or sealed chambers, which result in continuously reducing oxygen levels until anoxia.[44, 74] Other reasons for the discrepancies observed in the differentiation potential include different serum concentrations and preculture conditions used, duration of hypoxia, and whether differentiation was assessed under hypoxic conditions or in atmospheric oxygen. Source of the MSCs might have a large effect on the outcome of such experiments. Donor age, for example, can have a clear effect, as young MSCs were reported to respond better to anoxia and were more capable of continuing upregulation of angiogenic genes after reoxygenation than old MSCs.[106] Regardless of discrepancies, it is clear that oxygen tension is a vital consideration in MSC biology and application. It is important to note that almost all pO2 measurements, if made, were in the gas phase and will not accurately represent the oxygen tension experienced by the cells. Due to

technical limitations (previously described in Refs.[19, 22]) it is difficult to determine the exact oxygen tension experienced by cells in culture. In monolayer culture the oxygen has to diffuse through the culture medium to reach the cells, which causes lower oxygen tensions near the cell surface than those controlled in the incubator. This is demonstrated by Fink et al., who showed a pO2 reading of 0.14% at the cell surface when the oxygen tension was 1% in the gas phase. Similarly, the pericellular oxygen tension was 15.1% in a culture under ambient air (21% pO2).[72] This problem is aggravated in three-dimensional culture where diffusion limitations are even more severe, especially when matrix production increases the construct's density. Although the discrepancy between pO2 in the gas phase and liquid phase is obvious, it is important to note that it depends on several factors like the height of the medium above the cells, temperature, and mixing. Therefore, oxygen tension control (or at least measurements) should be performed in the liquid phase to ensure that the given oxygen tension accurately reflects the pO2 experienced by the cells. Additionally, large fluctuations in oxygen tension can occur if standard culture procedures are not performed under controlled oxygen tension. Consequently, medium changes and passaging should be done using preequilibrated medium in dedicated pO2-controlled flow hoods to avoid fluctuations in pO2 during culture, since all these factors can contribute to the variations and contradictions found within the literature. As a result, the culture system and control method always need to be considered when interpreting data on oxygen tension. In the future, a standardized protocol for hypoxic culture might lead to more consensus on the effect of hypoxia in MSC culture.

Conclusion

In this review we discussed the role of hypoxia on MSCs for regenerative medicine. Hypoxic culture of MSCs will play an important role in future strategies for regenerative medicine because it can enhance proliferation and differentiation. Additionally, hypoxic preconditioning of MSCs before implantation and associated hypoxia-conditioned medium can improve cell survival in vivo, which has significant effects on long-term effectiveness of MSC therapy. As the pathways involved in the various aspects of MSC

culture are unraveled, optimal protocols for regenerative cell therapy will move ever closer. Manipulating cell behavior through control of oxygen tension will have a prominent place in these protocols. The ability to culture cells and enhance their rate of proliferation, differentiation potential, and in vivo survival simply by lowering oxygen tension offers exciting new potential for the field of tissue engineering. The possibility of positively altering cell behavior on this scale without the use of additional growth factors, viruses, or other nonclinically relevant agents should attract the interest of many researchers aiming to bring regenerative medicine closer to the clinic.

Acknowledgements

This work was funded by the Dutch Program Tissue Engineering (Project No. RGT.6738), the European Community's Seventh Framework Programme (FP7/2007-2013), Marie Curie Intra European Fellowship (under grant agreement No. 221345), and the Dutch Arthritis Association (Reumafonds). The authors would like to thank Femke Verseijden for critical comments on this review.

References

1. Friedenstein, A.J., R.K. Chailakhjan, and K.S. Lalykina, The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet, 1970. 3(4): p. 393-403.
2. Friedenstein, A.J., R.K. Chailakhyan, and U.V. Gerasimov, Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet, 1987. 20(3): p. 263-72.
3. Friedenstein, A.J., et al., Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol, 1974. 2(2): p. 83-92.
4. Friedenstein, A.J., et al., Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation, 1968. 6(2): p. 230-47.
5. Friedenstein, A.J., S. Piatetzky, II, and K.V. Petrakova, Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol, 1966. 16(3): p. 381-90.
6. Caplan, A.I., Mesenchymal stem cells. J Orthop Res, 1991. 9(5): p. 641-50.
7. Aubin, J.E., Osteogenic cell differentiation, in Bone Engineering, J.E. Davies, Editor. 2000, Em Squared Inc.: Toronto. p. 19-30.
8. Aubin, J.E., et al., Osteoblast and chondroblast differentiation. Bone, 1995. 17(2 Suppl): p. 77S-83S.
9. Barry, F.P. and J.M. Murphy, Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol, 2004. 36(4): p. 568-84.
10. Caplan, A.I. and J.E. Dennis, Mesenchymal stem cells as trophic mediators. J Cell Biochem, 2006. 98(5): p. 1076-84.
11. Herzog, E.L., L. Chai, and D.S. Krause, Plasticity of marrow-derived stem cells. Blood, 2003. 102(10): p. 3483-93.
12. Jaiswal, N., et al., Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem, 1997. 64(2): p. 295-312.
13. Kadiyala, S., et al., Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant, 1997. 6(2): p. 125-34.

14. Mackay, A.M., et al., Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng, 1998. 4(4): p. 415-28.
15. Maniatopoulos, C., J. Sodek, and A.H. Melcher, Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res, 1988. 254(2): p. 317-30.
16. Mulholland, D.J., et al., Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? Endocr Rev, 2005. 26(7): p. 898-915.
17. Pittenger, M.F., et al., Multilineage potential of adult human mesenchymal stem cells. Science, 1999. 284(5411): p. 143-7.
18. Pittenger, M.F. and B.J. Martin, Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res, 2004. 95(1): p. 9-20.
19. Csete, M., Oxygen in the cultivation of stem cells. Ann N Y Acad Sci, 2005. 1049: p. 1-8.
20. Lennon, D.P., J.M. Edmison, and A.I. Caplan, Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol, 2001. 187(3): p. 345-55.
21. Cipolleschi, M.G., P. Dello Sbarba, and M. Olivotto, The role of hypoxia in the maintenance of hematopoietic stem cells. Blood, 1993. 82(7): p. 2031-7.
22. Ma, T., et al., Hypoxia and stem cell-based engineering of mesenchymal tissues. Biotechnol Prog, 2009. 25(1): p. 32-42.
23. Ivanovic, Z., Hypoxia or in situ normoxia: The stem cell paradigm. J Cell Physiol, 2009. 219(2): p. 271-5.
24. Cooper, P.D., A.M. Burt, and J.N. Wilson, Critical effect of oxygen tension on rate of growth of animal cells in continuous suspended culture. Nature, 1958. 182(4648): p. 1508-9.
25. Zwartouw, H.T. and J.C. Westwood, Factors affecting growth and glycolysis in tissue culture. Br J Exp Pathol, 1958. 39(5): p. 529-39.
26. Farrell, E., et al., Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Eng Part C Methods, 2009. 15(2): p. 285-95.
27. Meijer, G.J., et al., Cell based bone tissue engineering in jaw defects. Biomaterials, 2008. 29(21): p. 3053-61.
28. Nesselmann, C., et al., Mesenchymal stem cells and cardiac repair. J

Cell Mol Med, 2008. 12(5B): p. 1795-810.
29. Rouwkema, J., N.C. Rivron, and C.A. van Blitterswijk, Vascularization in tissue engineering. Trends Biotechnol, 2008. 26(8): p. 434-41.
30. Tang, Y.L., et al., Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. J Am Coll Cardiol, 2005. 46(7): p. 1339-50.
31. Verseijden, F., et al., Angiogenic capacity of human adipose-derived stromal cells during adipogenic differentiation: an in vitro study. Tissue Eng Part A, 2009. 15(2): p. 445-52.
32. Xu, R.X., et al., Mesenchymal stem cells promote cardiomyocyte hypertrophy in vitro through hypoxia-induced paracrine mechanisms. Clin Exp Pharmacol Physiol, 2009. 36(2): p. 176-80.
33. Reinecke, H. and C.E. Murry, Cell grafting for cardiac repair. Methods Mol Biol, 2003. 219: p. 97-112.
34. Toma, C., et al., Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation, 2002. 105(1): p. 93-8.
35. Zhang, M., et al., Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol, 2001. 33(5): p. 907-21.
36. Shintani, S., et al., Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation, 2001. 103(23): p. 2776-9.
37. Sobajima, S., et al., Feasibility of a stem cell therapy for intervertebral disc degeneration. Spine J, 2008. 8(6): p. 888-96.
38. Zhang, Y.G., et al., Bone mesenchymal stem cells transplanted into rabbit intervertebral discs can increase proteoglycans. Clin Orthop Relat Res, 2005(430): p. 219-26.
39. Murphy, J.M., et al., Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum, 2003. 48(12): p. 3464-74.
40. Zhu, W., et al., Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. Stem Cells, 2006. 24(2): p. 416-25.
41. Potier, E., et al., Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. Tissue Eng, 2007. 13(6): p. 1325-31.
42. Grayson, W.L., et al., Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. J Cell Physiol, 2006. 207(2):

p. 331-9.
43. Mylotte, L.A., et al., Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. Stem Cells, 2008. 26(5): p. 1325-36.
44. Potier, E., et al., Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone, 2007. 40(4): p. 1078-87.
45. Jaakkola, P., et al., Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science, 2001. 292(5516): p. 468-72.
46. Kanichai, M., et al., Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. J Cell Physiol, 2008. 216(3): p. 708-15.
47. Lord-Dufour, S., et al., Evidence for transcriptional regulation of the glucose-6-phosphate transporter by HIF-1alpha: Targeting G6PT with mumbaistatin analogs in hypoxic mesenchymal stromal cells. Stem Cells, 2009. 27(3): p. 489-97.
48. Hu, X., et al., Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg, 2008. 135(4): p. 799-808.
49. Theus, M.H., et al., In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. Exp Neurol, 2008. 210(2): p. 656-70.
50. Gui, C., et al., Heregulin protects mesenchymal stem cells from serum deprivation and hypoxia-induced apoptosis. Mol Cell Biochem, 2007. 305(1-2): p. 171-8.
51. Hung, S.C., et al., Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells, 2007. 25(9): p. 2363-70.
52. Rosova, I., et al., Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells, 2008. 26(8): p. 2173-82.
53. Datta, S.R., A. Brunet, and M.E. Greenberg, Cellular survival: a play in

three Akts. Genes Dev, 1999. 13(22): p. 2905-27.
54. Mangi, A.A., et al., Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med, 2003. 9(9): p. 1195-201.
55. Gnecchi, M., et al., Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J, 2006. 20(6): p. 661-9.
56. Xu, R., et al., Lovastatin protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt and ERK1/2. J Cell Biochem, 2008. 103(1): p. 256-69.
57. Liu, X., et al., Lysophosphatidic acid protects mesenchymal stem cells against ischemia-induced apoptosis in vivo. Stem Cells Dev, 2009. 18(7): p. 947-54.
58. Liu, X.H., et al., Therapeutic potential of angiogenin modified mesenchymal stem cells: angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. Microvasc Res, 2008. 76(1): p. 23-30.
59. Li, W., et al., Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells, 2007. 25(8): p. 2118-27.
60. Grayson, W.L., et al., Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochem Biophys Res Commun, 2007. 358(3): p. 948-53.
61. D'Ippolito, G., et al., Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone, 2006. 39(3): p. 513-22.
62. Carrancio, S., et al., Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. Exp Hematol, 2008. 36(8): p. 1014-21.
63. Ren, H., et al., Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. Biochem Biophys Res Commun, 2006. 347(1): p. 12-21.
64. Martin-Rendon, E., et al., Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia. Stem Cells, 2007. 25(4): p. 1003-12.
65. Hung, S.C., et al., Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their

engraftment in vivo. PLoS One, 2007. 2(5): p. e416.
66. Yun, S.P., et al., Role of HIF-1alpha and VEGF in human mesenchymal stem cell proliferation by 17beta-estradiol: involvement of PKC, PI3K/Akt, and MAPKs. Am J Physiol Cell Physiol, 2009. 296(2): p. C317-26.
67. Salim, A., et al., Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. J Biol Chem, 2004. 279(38): p. 40007-16.
68. Muller, I., et al., Secretion of angiogenic proteins by human multipotent mesenchymal stromal cells and their clinical potential in the treatment of avascular osteonecrosis. Leukemia, 2008. 22(11): p. 2054-61.
69. Robins, J.C., et al., Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. Bone, 2005. 37(3): p. 313-22.
70. Scherer, K., et al., The influence of oxygen and hydrostatic pressure on articular chondrocytes and adherent bone marrow cells in vitro. Biorheology, 2004. 41(3-4): p. 323-33.
71. Hirao, M., et al., Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. J Biol Chem, 2006. 281(41): p. 31079-92.
72. Fink, T., et al., Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. Stem Cells, 2004. 22(7): p. 1346-55.
73. Iwase, T., et al., Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc Res, 2005. 66(3): p. 543-51.
74. Annabi, B., et al., Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. Stem Cells, 2003. 21(3): p. 337-47.
75. Hai-Jiang, W., D. Xin-Na, and D. Hui-Jun, Expansion of hematopoietic stem/progenitor cells. Am J Hematol, 2008. 83(12): p. 922-6.
76. Chen, L., et al., Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One, 2008. 3(4): p. e1886.
77. Li, T.S., et al., Myocardial repair achieved by the intramyocardial implantation of adult cardiomyocytes in combination with bone marrow cells. Cell Transplant, 2008. 17(6): p. 695-703.
78. Hokari, M., et al., Bone marrow stromal cells protect and repair

damaged neurons through multiple mechanisms. J Neurosci Res, 2008. 86(5): p. 1024-35.
79. Aggarwal, S. and M.F. Pittenger, Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood, 2005. 105(4): p. 1815-22.
80. Krampera, M., et al., Regenerative and immunomodulatory potential of mesenchymal stem cells. Curr Opin Pharmacol, 2006. 6(4): p. 435-41.
81. Hoogduijn, M.J., et al., Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev, 2007. 16(4): p. 597-604.
82. Crop, M., et al., Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int, 2009. 22(4): p. 365-76.
83. Krampera, M., et al., Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood, 2003. 101(9): p. 3722-9.
84. Groh, M.E., et al., Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. Exp Hematol, 2005. 33(8): p. 928-34.
85. Ohnishi, S., et al., Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells, 2007. 25(5): p. 1166-77.
86. Wu, E.H., et al., [Effect of hypoxia on the gene profile of human bone marrow-derived mesenchymal stem cells]. Sheng Li Xue Bao, 2007. 59(2): p. 227-32.
87. Kinnaird, T., et al., Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res, 2004. 94(5): p. 678-85.
88. Muir, C., et al., Hypoxia increases VEGF-A production by prostate cancer and bone marrow stromal cells and initiates paracrine activation of bone marrow endothelial cells. Clin Exp Metastasis, 2006. 23(1): p. 75-86.
89. Wang, Y., Y. Deng, and G.Q. Zhou, SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. Brain Res, 2008. 1195: p. 104-12.
90. Ryan, J.M., et al., Interferon-gamma does not break, but promotes the

immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol, 2007. 149(2): p. 353-63.
91. Crisostomo, P.R., et al., Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. Am J Physiol Cell Physiol, 2008. 294(3): p. C675-82.
92. Mao, X., et al., Angiogenic potency of bone marrow stromal cells improved by ex vivo hypoxia prestimulation. J Huazhong Univ Sci Technolog Med Sci, 2004. 24(6): p. 566-8.
93. Wang, J.A., et al., Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. Acta Pharmacol Sin, 2008. 29(1): p. 74-82.
94. Rochefort, G.Y., et al., Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells, 2006. 24(10): p. 2202-8.
95. Bonnet, P., et al., Chronic hypoxia induces nonreversible right ventricle dysfunction and dysplasia in rats. Am J Physiol Heart Circ Physiol, 2004. 287(3): p. H1023-8.
96. Hyvelin, J.M., et al., Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. Circ Res, 2005. 97(2): p. 185-91.
97. Ben-Yosef, Y., et al., Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res, 2002. 90(7): p. 784-91.
98. Ottino, P., et al., Hypoxia activates matrix metalloproteinase expression and the VEGF system in monkey choroid-retinal endothelial cells: Involvement of cytosolic phospholipase A2 activity. Mol Vis, 2004. 10: p. 341-50.
99. Barbash, I.M., et al., Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation, 2003. 108(7): p. 863-8.
100. Ji, J.F., et al., Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. Neurosci Lett, 2004. 355(3): p. 236-40.
101. Ma, Q., et al., Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A, 1998. 95(16): p. 9448-53.

102. Tepper, O.M., et al., Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation, 2002. 106(22): p. 2781-6.
103. Askari, A.T., et al., Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet, 2003. 362(9385): p. 697-703.
104. Gerber, I. and I. ap Gwynn, Differentiation of rat osteoblast-like cells in monolayer and micromass cultures. Eur Cell Mater, 2002. 3: p. 19-30.
105. Son, B.R., et al., Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells, 2006. 24(5): p. 1254-64.
106. Jiang, S., et al., Transcriptional profiling of young and old mesenchymal stem cells in response to oxygen deprivation and reparability of the infarcted myocardium. J Mol Cell Cardiol, 2008. 44(3): p. 582-96.

7

Chapter 7

Discussion and conclusion

A successful, long-term treatment for cartilage defects and osteoarthritis is still a major unmet clinical need. Despite numerous attempts with various cell- and biomaterial based solutions, there currently is no treatment to truly restore cartilage functionality. Autologous chondrocyte implantation is still the gold standard cell-based solution, although alternatives are clinically available, such as INSTRUCT from CellCoTec. While the short-term success of these new approaches can be encouraging, long-term effects are often not superior to ACI. While ACI and matrix assisted chondrocyte implantation (MACI) provide significant improvements in histological and clinical outcome compared to microfracture [1], major advancement can yet be made by optimizing these procedures. Any cell based approach for cartilage repair benefits from using cells that are optimally suited to perform their function after reimplantation. To achieve this, it is imperative that the culture parameters in vitro are tailored to provide the best environment for cells. This thesis explored the impact that different parameters can have when attempting to redifferentiate chondrocytes or obtain cells with the best chondrogenic potential.

The chondrocyte residing in articular cartilage has long been acknowledged to have mechanosensitive properties [2-6]. Since cartilage is loaded and unloaded on a daily basis, it is not surprising that chondrocytes seem to be able to respond to deformation of their environment (and consequently themselves). Although normal, physiological loading is thought to be a prerequisite for the maintenance of a healthy cartilage joint [7], this is not so apparent for chondrocytes that have an altered physiological state. Chondrocytes from diseased joints, as well as those that have shifted their phenotype as a result of in-vitro culture expansion, respond differently to changes in their mechanical environment than chondrocytes from healthy cartilage. Chapter 2 shows that expansion of human articular chondrocytes clearly alters their response to mechanical stimulation. As a consequence, when evaluating the effects of mechanical loading on chondrocytes, not only does the mechanical environment have to be accurately defined, the cells themselves need to be properly characterized to be able to put results of loading experiments in a correct light.
Bioreactor technology is able to provide such an accurately defined mechanical environment, allowing them to simulate in-vivo processes.

However, the mechanical environment of the chondrocyte in vivo is complex and not defined solely by one type of loading. An intricate interplay of hydrostatic pressure, compressive loading and shear makes up the mechanical environment of the chondrocyte in articular cartilage [8, 9]. Various bioreactor designs have been employed to evaluate the effects of these loading conditions, either individually or a combination, using more complex systems. The effects of these loading conditions appear to be anabolic in terms in ECM production, but only where they concern physiological loading of primary chondrocytes from healthy tissue [2], as high impact loading is regarded to be detrimental. In fact, the effects of physiological loading even vary with the location of origin of the healthy chondrocytes. Chondrocytes harvested from the superficial zone, for example, respond differently to shear forces than those residing in the deeper zones of cartilage [10], which can be attributed to their exposure to these forces in vivo. With different responses to loading occurring even within the population of healthy chondrocytes, it should not be surprising that these responses also differ when chondrocytes are harvested from diseased tissue.

It is well established that loading representing non-physiological, injurious type situations can induce OA-like changes in primary chondrocytes [11-14].What exactly constitutes a non-physiological load depends again on the origin of the chondrocyte. The knee joint is loaded on a daily basis, but the forces that it has to withstand vary with location. For this reason, chondrocytes for ACI procedures are harvested from cartilage from non-loadbearing regions of the tissue. The ability of these chondrocytes to cope with a more challenging loading environment is further compromised by expansion culture, as shown in Chapter 2 of this thesis. Mechanical loading is often used to direct the differentiation of chondrocytes in three-dimensional constructs. However, it appears difficult to establish the optimal loading protocol that results in the highest anabolic response for such constructs, especially when that response depends so heavily on the exact phenotype of the cell.

Looking at the literature on mechanical loading of 3D construct, it is clear exactly how difficult it is to find a protocol that accurately represents the

in-vivo situation. Mechanical loading in vivo can be divided into several components. Hydrostatic pressure, compressive loading and shear all play a role in the daily loading of cartilage and as such should all be considered when attempting to mimic the physiological situation using bioreactors.

Hydrostatic pressure might be the easiest form of loading to mimic in vitro. A range of 7 to 10 MPa is considered to be physiological and intermittent loading in this range has been shown to promote matrix synthesis in various experimental designs [15]. However, increased apoptosis was found in intermittently loaded constructs that made use of osteoarthritic human chondrocytes[16], again highlighting the difficulty of translating studies across species and between pathological states.

Uniaxial compressive loading using bioreactor systems can mimic the direct contact between joint surfaces during gait. For articular cartilage of the major weight bearing joints (such as knees and ankles), average loadings of approximately 0.5 to 7.7 MPa and average compression amplitudes of more than 13% have been measured during normal daily movements [15, 17-19]. The way this loading is sensed by the cells depends heavily on the type of material that is used to construct the 3D environment and the way the cells interact with this material, but several papers for both chondrocytes [3, 20, 21] and MSCs [22-24] claim a positive effect on chondrogenic gene expression and GAG synthesis for 10% compression at 1 Hz.

Efforts have been made to more accurately mimic the in-vivo situation using complex bioreactor systems that can apply several forms of loading simultaneously. A combination of compression and shear can be used to mimic deformation during various stages of joint loading, but it is difficult to establish the degree to which this loading represents in vivo processes. Deformations and loads at different locations (e.g. medial vs. lateral tibial plateau) vary even in the same load cycle [25, 26]. Therefore, recapitulating in vivo loading using bioreactor technology appears too complex for regenerative medicine approaches. While the mimicking approach can serve a purpose in predicting (patho)physiological processes using well-defined chondrocytes/cartilage, the complexity limits the uses for

therapeutic applications.

The environment of the chondrocyte is not only mechanically challenging. Chondrocytes are exposed to environmental challenges in both physiological and pathophysiological conditions. Due to the lack of vascularization in cartilage, parameters such as oxygen tension and pH are uncommonly low even in a healthy joint. Chondrocytes are exceptionally adapted to this hostile environment, but being able to survive in an oxygen-deprived, acidic environment is only the first step. Within this environment, the cells need to maintain a functional extracellular matrix. While this might already be challenging in a healthy joint, joint disease such as osteoarthritis will aggravate the situation by lowering these parameters even further. While, or because, they are challenged with an increasingly hostile environment, chondrocytes undergo phenotypical changes that alter their ability to cope with this environment. In this thesis we have shown how new tools in cell culture can shed light on the way these changes affect fundamental aspects of chondrocyte physiology.

Hypoxia is by far the most investigated environmental parameter is cartilage, with pH and osmolarity receiving only limited attention. There is ample evidence that culture at low oxygen tensions (i.e. oxygen tensions that more closely resemble the in vivo situation than standard culture conditions) can have a positive effect on the ECM production of both primary chondrocytes [27, 28] and mesenchymal stem cells [29, 30]. Hypoxia can also be employed to steer dedifferentiated chondrocytes (due to expansion) back towards a chondrocyte phenotype, which can be used to prime cells before implantation. Again it is important to distinguish between the effect that hypoxia has on healthy, primary cells and on cells that are phenotypically different. Describing these differences will aid in understanding the process of dedifferentiation. Some headway has been made in understanding the biology behind the positive effects of hypoxic culture [31-33], but mechanisms remain to be fully unraveled. Many effects appear to be related to HIF-2α dependent signaling via SOX9 [33], but other processes occur independent of this pathway. Moreover, several signaling pathways might work in concert. Chapter 4 of this thesis highlights one particular cellular mechanism of hypoxia in redifferentiating

chondrocytes that plays a role in ECM expression. TGF-β is critically involved in various cartilage-related processes. Notably, TGF-βs and their receptors play a role in chondrocyte proliferation and differentiation [34-38], maintain articular cartilage in a prehypertrophic state by repressing terminal differentiation [39], and direct undifferentiated MSCs towards the chondrogenic phenotype [40, 41]. Hypoxia was already known to affect the expression of TGF-β(2) in other cell types [42, 43] and in healthy bovine chondrocytes during hypoxia/reoxygenation [44]. In redifferentiating chondrocytes the expression of TGF-β2 and its type I receptors was upregulated under hypoxia, resulting in increased expression of COL2 and SOX9, important markers for the chondrocyte phenotype. Clearly, this cell signaling pathway could be manipulated using an environmental stimulus. Identifying the effect of environmental stimuli, not only on ECM products, but also on cell signaling pathways that govern their expression, will reveal new avenues to enhance the outcome of (cell based) therapies.

However, limiting the degree of dedifferentiation during expansion, rather than enhancing consecutive redifferentiation, should be a prime concern for cell based therapies for cartilage repair that require large cell numbers. Controlling environmental parameters during the expansion culture can help achieve this goal. Chapter 5 demonstrates the effect that elevated osmolarity has on the expression of chondrocyte-specific markers during expansion culture. In addition, employing low oxygen tensions only during the expansion phase also improves the chondrogenic potential for MSCs and stimulates redifferentiation of chondrocytes [45-47]. It would be interesting to examine how expansion under these altered environment affect the cell's response to mechanical stimuli. In this case, mechanical loading using bioreactor technology can be used as a screening tool to predict the cell's function in vivo after reimplantation, rather than a way of priming cells before implantation.
When investigating the effect of these parameters, it is important to keep in mind that, as demonstrated in Chapter 3, effects of the different parameters depend on each other. Other examples for these interactions can also be found in literature. For example, the ability to regulate intracellular pH recovery in articular chondrocytes is affected by hypoxia [48] and a combination of low pH and high osmolarity had negative effects on the

survival and expression of ECM components in adipose derived stem cells in a model for intervertebral disk regeneration [49]. These findings make a strong case for the need to describe the total environment during cell culture, or when attempting to model pathophysiological conditions in vitro. Similar to the biomechanical environment, the physiochemical environment is complex. However, unlike the biomechanical environment, the physiochemical environment is relatively easy to manipulate. Innovative bioreactor systems, such as those described in Chapter 3, allow for the investigation of effects of the culture environment in a controlled manner. The ability to accurately control all relevant culture parameters individually is indispensable when investigating isolated effects. Semi-high throughput bioreactors have the added advantage of screening multiple conditions within one experiment, something most bioreactors lack due to their inherent complexity. Coupling this (semi-)high throughput screening with Design of Experiment analytical approaches creates a powerful tool to examine cell responses to and interactions of the various environmental parameters.

It is clear from this work and literature that various types of stimulation can be used to enhance the chondrogenic potential of cells that are used for therapy in cartilage repair. Bioreactors that control the mechanical and/or physiochemical environment of cells in culture can direct cell function. Elaborate systems can be envisioned that incorporate all these stimuli into one bioreactor for an optimal pre-culture of cells before implantation. However, the road to clinical application of cell based therapies is not only challenging from a scientific view point, in the end any therapy needs to be practicable. Consequently, logistic and regulatory considerations play a large role in the translation of fundamental research to clinical practice. The need for cell expansion and especially the use of cell-scaffold interactions complicates both. The logistics involved in transportation of cells and operator involvement for elaborate protocols increase cost, risk and efficacy of such approaches (cell expansion is considered an extensive manipulation according to EU directive 1394/2007, which causes cells produced for such therapies to be labeled as Advanced Therapy Medicinal Products (ATMPs)). Minimizing the complexity of the culture procedure while maximizing the chondrogenic potential favors the

use of environmental parameter control rather than mechanical loading, due to the intricate nature of the latter. Therefore, rather than employing mechanical loading bioreactors as a tool to direct extracellular matrix production, they might be used as a screening tool to predict the in vivo function after reimplantation.

The use of environmental parameters as a method to direct cell function, beyond chondrocytes, reveals its potential for other cell types. Chapter 6 explores the use of hypoxia to direct MSC differentiation, survival, proliferation and trophic factor release. Current literature does not yet give a clear picture of its effect, but it is evident that oxygen tension is an important aspect in MSC culture, since it can affect the potency of a cell therapy product, whatever the mechanism of action (immunomodulation, secretion of factors, or direct differentiation). This is an important consideration, since the use of MSCs for cell therapy applications is becoming more wide-spread, as the number of clinical trials increases (from clinicaltrials.gov). Since in-vitro culture, which is needed to reach the clinical cell numbers needed for such therapies, induces phenotypical changes that can affect the therapeutic potential [50], and requires optimization for specific applications. Depending on the mechanism of action of the cell therapy, it might be required to recapitulate the original stem cell niche [51], most likely using bioreactors for environmental control, to ensure the cells reach/maintain their full therapeutic potential. However, maintaining cells in physiological environments is not the only use for cell manipulation. Stunningly, using environmental manipulation, Obokata et al. [52, 53] showed that "epigenetic fate determination of mammalian cells can be markedly converted in a context-dependent manner by strong environmental cues" [52]. Their use of parameters that lie outside the physiological range, such as the use of sub-lethal low pH treatment, to reprogram cells to a pluripotent state, truly reveals the untapped potential of environmental stimulation. The derivation of pluripotent cells from not only purified lymphocytes, but various other cell types including chondrocytes, shows that fate determination can be converted. This has obvious implications for future lines of research in which environmental control will play a significant role, since cells with a pluripotent phenotype are currently only available from ethically sensitive material (embryonic

stem cells) or through laborious manipulation (induced pluripotent stem cells). Simplified creation of cell lines through environmental stimulation could accelerate research and subsequent development of therapy.

It is clear from that environmental stimulation can play a significant role in both experimentation, to understand (patho)physiological processes, and production of cell therapies, to maximize therapeutic potential. For chondrocytes, this thesis describes effects of mechanical stimulation, hypoxia, pH and osmolarity, in an attempt to further optimize chondrocyte culture. Future work, exploring combined effects and interdependencies, should lead to further optimization of parameters for (chondrocyte) cell culture.

References

1. Perera, J.R., P.D. Gikas, and G. Bentley, The present state of treatments for articular cartilage defects in the knee. Ann R Coll Surg Engl, 2012. 94(6): p. 381-7.
2. Buschmann, M.D., et al., Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci, 1995. 108 (Pt 4): p. 1497-508.
3. Chowdhury, T.T., et al., Temporal regulation of chondrocyte metabolism in agarose constructs subjected to dynamic compression. Arch Biochem Biophys, 2003. 417(1): p. 105-11.
4. Sah, R.L., et al., Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res, 1989. 7(5): p. 619-36.
5. Jones, I.L., A. Klamfeldt, and T. Sandstrom, The effect of continuous mechanical pressure upon the turnover of articular cartilage proteoglycans in vitro. Clin Orthop Relat Res, 1982(165): p. 283-9.
6. Grodzinsky, A.J., et al., Cartilage tissue remodeling in response to mechanical forces. Annu Rev Biomed Eng, 2000. 2: p. 691-713.
7. Vanwanseele, B., et al., Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement. Arthritis Rheum, 2002. 46(8): p. 2073-8.
8. Buckwalter, J.A., H.J. Mankin, and A.J. Grodzinsky, Articular cartilage and osteoarthritis. Instr Course Lect, 2005. 54: p. 465-80.
9. Guilak, F., Biomechanical factors in osteoarthritis. Best Pract Res Clin Rheumatol, 2011. 25(6): p. 815-23.
10. Li, Z., et al., Different response of articular chondrocyte subpopulations to surface motion. Osteoarthritis Cartilage, 2007. 15(9): p. 1034-41.
11. Natoli, R.M., C.C. Scott, and K.A. Athanasiou, Temporal effects of impact on articular cartilage cell death, gene expression, matrix biochemistry, and biomechanics. Ann Biomed Eng, 2008. 36(5): p. 780-92.
12. Buckwalter, J.A., J.A. Martin, and T.D. Brown, Perspectives on chondrocyte mechanobiology and osteoarthritis. Biorheology, 2006. 43(3-4): p. 603-9.
13. Borrelli, J., Jr., et al., Induction of chondrocyte apoptosis following impact load. J Orthop Trauma, 2003. 17(9): p. 635-41.

14. Jeffrey, J.E., D.W. Gregory, and R.M. Aspden, Matrix damage and chondrocyte viability following a single impact load on articular cartilage. Arch Biochem Biophys, 1995. 322(1): p. 87-96.
15. Elder, B.D. and K.A. Athanasiou, Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. Tissue Eng Part B Rev, 2009. 15(1): p. 43-53.
16. Wenger, R., et al., Hydrostatic pressure increases apoptosis in cartilage-constructs produced from human osteoarthritic chondrocytes. Front Biosci, 2006. 11: p. 1690-5.
17. Afoke, N.Y., P.D. Byers, and W.C. Hutton, Contact pressures in the human hip joint. J Bone Joint Surg Br, 1987. 69(4): p. 536-41.
18. Mow, V.C. and C.C. Wang, Some bioengineering considerations for tissue engineering of articular cartilage. Clin Orthop Relat Res, 1999(367 Suppl): p. S204-23.
19. von Eisenhart, R., et al., Quantitative determination of joint incongruity and pressure distribution during simulated gait and cartilage thickness in the human hip joint. J Orthop Res, 1999. 17(4): p. 532-9.
20. Mauck, R.L., et al., Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng, 2000. 122(3): p. 252-60.
21. Mauck, R.L., et al., The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis Cartilage, 2003. 11(12): p. 879-90.
22. Huang, C.Y., et al., Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. Stem Cells, 2004. 22(3): p. 313-23.
23. Mouw, J.K., et al., Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. Stem Cells, 2007. 25(3): p. 655-63.
24. Terraciano, V., et al., Differential response of adult and embryonic mesenchymal progenitor cells to mechanical compression in hydrogels. Stem Cells, 2007. 25(11): p. 2730-8.
25. Grad, S., et al., Physical stimulation of chondrogenic cells in vitro: a review. Clin Orthop Relat Res, 2011. 469(10): p. 2764-72.
26. Hosseini, A., et al., In-vivo time-dependent articular cartilage contact behavior of the tibiofemoral joint. Osteoarthritis Cartilage, 2010. 18(7): p.

909-16.
27. Coyle, C.H., N.J. Izzo, and C.R. Chu, Sustained hypoxia enhances chondrocyte matrix synthesis. J Orthop Res, 2009. 27(6): p. 793-9.
28. Pfander, D., et al., HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. J Cell Sci, 2003. 116(Pt 9): p. 1819-26.
29. Meretoja, V.V., et al., The effect of hypoxia on the chondrogenic differentiation of co-cultured articular chondrocytes and mesenchymal stem cells in scaffolds. Biomaterials, 2013. 34(17): p. 4266-73.
30. Hirao, M., et al., Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. J Biol Chem, 2006. 281(41): p. 31079-92.
31. Aro, E., et al., Hypoxia-inducible factor-1 (HIF-1) but not HIF-2 is essential for hypoxic induction of collagen prolyl 4-hydroxylases in primary newborn mouse epiphyseal growth plate chondrocytes. J Biol Chem, 2012. 287(44): p. 37134-44.
32. Lafont, J.E., et al., Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. J Biol Chem, 2008. 283(8): p. 4778-86.
33. Lafont, J.E., S. Talma, and C.L. Murphy, Hypoxia-inducible factor 2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis Rheum, 2007. 56(10): p. 3297-306.
34. Boumediene, K., et al., Modulation of rabbit articular chondrocyte (RAC) proliferation by TGF-beta isoforms. Cell Prolif, 1995. 28(4): p. 221-34.
35. Okazaki, R., et al., Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. Ann Rheum Dis, 1996. 55(3): p. 181-6.
36. de Haart, M., et al., Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes. Acta Orthop Scand, 1999. 70(1): p. 55-61.
37. Yonekura, A., et al., Transforming growth factor-beta stimulates articular chondrocyte cell growth through p44/42 MAP kinase (ERK) activation. Endocr J, 1999. 46(4): p. 545-53.
38. Smith, P., et al., Genetic enhancement of matrix synthesis by articular chondrocytes: comparison of different growth factor genes in the presence and absence of interleukin-1. Arthritis Rheum, 2000. 43(5): p. 1156-64.

39. Yang, X., et al., TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol, 2001. 153(1): p. 35-46.
40. Lee, J.W., et al., Chondrogenic differentiation of mesenchymal stem cells and its clinical applications. Yonsei Med J, 2004. 45 Suppl: p. 41-7.
41. Fukumoto, T., et al., Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis Cartilage, 2003. 11(1): p. 55-64.
42. Akman, H.O., et al., Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. Blood, 2001. 98(12): p. 3324-31.
43. Zhang, H., et al., Cellular response to hypoxia involves signaling via Smad proteins. Blood, 2003. 101(6): p. 2253-60.
44. Martin, G., et al., Effect of hypoxia and reoxygenation on gene expression and response to interleukin-1 in cultured articular chondrocytes. Arthritis Rheum, 2004. 50(11): p. 3549-60.
45. Adesida, A.B., A. Mulet-Sierra, and N.M. Jomha, Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells. Stem Cell Res Ther, 2012. 3(2): p. 9.
46. Egli, R.J., et al., Hypoxic expansion promotes the chondrogenic potential of articular chondrocytes. J Orthop Res, 2008. 26(7): p. 977-85.
47. Henderson, J.H., et al., Low oxygen tension during incubation periods of chondrocyte expansion is sufficient to enhance postexpansion chondrogenesis. Tissue Eng Part A, 2010. 16(5): p. 1585-93.
48. Milner, P.I., et al., Growth factor regulation of intracellular pH homeostasis under hypoxic conditions in isolated equine articular chondrocytes. J Orthop Res, 2013. 31(2): p. 197-203.
49. Liang, C., et al., Responses of human adipose-derived mesenchymal stem cells to chemical microenvironment of the intervertebral disc. J Transl Med, 2012. 10: p. 49.
50. Bara, J.J., et al., Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: Implications for basic research and the clinic. Stem Cells, 2014.
51. Augello, A., T.B. Kurth, and C. De Bari, Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur Cell

Mater, 2010. 20: p. 121-33.
52. Obokata, H., et al., Stimulus-triggered fate conversion of somatic cells into pluripotency. Nature, 2014. 505(7485): p. 641-7.
53. Obokata, H., et al., Bidirectional developmental potential in reprogrammed cells with acquired pluripotency. Nature, 2014. 505(7485): p. 676-80.

8

Chapter 8

Nederlandse samenvatting

Gewrichtskraakbeen is een glad, wit weefsel dat zorg draagt voor de soepele en wrijvingsloze beweging van gewrichten. Bovendien functioneert het als een schokdemper die bij belasting de krachten gelijkmatig verdeelt over onderliggende botten.

Gewrichtskraakbeen bestaat voor het grootste deel uit relatief weinig componenten. Glycosaminoglycanen zijn negatief geladen en trekken daarom veel water aan, wat ervoor zorgt dat het weefsel zwelt. Kraakbeen bestaat dan ook voor een groot deel uit water. Deze zwelling wordt voornamelijk tegengegaan door de aanwezigheid van collageenvezels (type II). Het weefsel wordt bovendien onderhouden door slecht één celtype, de chondrocyt. Ondanks dat gewrichtskraakbeen uit zo weinig componenten bestaat is het lichaam niet goed in staat het te repareren. Het is daarom ook een weefsel dat veel aandacht heeft in het veld van tissue engineering dat gebruikt maakt van een combinatie van cellen, (bio) materialen en groeifactoren om zo buiten het lichaam een vervangend weefsel te produceren.

Er zijn veel manier om celgroei in een kweekomgeving te beïnvloeden. Eén van die manieren is om die kweekomgeving zelf te veranderen. Hiervoor kunnen bioreactoren gebruikt worden. Met behulp van deze apparaten kunnen cellen mechanisch belast worden, of kan de zuurstofspanning en pH van de kweekongeving nauwkeurig gecontrolleerd worden. Met behulp van deze manipulatie wordt celgedrag beïnvloed, waardoor een beter kweekresultaat verkregen kan worden.

In het lichaam wordt kraakbeen constant belast door dagelijkse bewegingen. In gezond kraakbeen helpt deze belasting bij het in stand houden van de integriteit van het weefsel; chondrocyten reageren op de belasting door structurele eiwitten (zoals proteoglycanen en type II collageen (COL2)) aan te maken. Chondrocyten uit osteoarthrotisch kraakbeen, of chondrocyten die in vitro gekweekt zijn, reageren echter anders op deze belasting. In hoofdstuk 2 van dit proefschift wordt op genexpressie niveau gekeken naar de reactie van chondrocyten op belasting. Wanneer chondrocyten uit osteoarthrotisch kraakbeen opgekweekt worden en vervolgens belast, dan is de en expressie van de belangrijkste structurele eiwitten, AGCN en

COL2, verlaagd, terwijl de expressie van degraderende enzymen, MMP1 en -3, juist verhoogd is. In tegenstelling tot primaire chondrocyten, die een meer anabole respons lieten zien met een opregulatie van de genexpressie van AGCN en COL2. Gedeeltelijke redifferentiatie zorgt ervoor dat de genexpressie van AGCN en COL2 als gevolg van mechanische belasting minder verlaagd is. De resultaten van deze studie hebben implicaties voor het expansieproces dat gebruikt wordt in autologe chondrocyt transplantatie alsmede het behandelplan van patiënten die behandeld zijn met geëxpandeerde chondrocyten.

Gewrichtskraakbeen bevat geen bloedvaten voor de aanvoer van zuurstof en de afvoer van afvalstoffen. De chondrocyten bevinden zicht daarom in een omgeving waar de zuurstofspanning (pO2) en pH lager zijn dan normaal (dwz dan in plasma). Hoofdstuk 3 onderzoekt de invloed die deze omgevingsparameters hebben op chondrocyten in kweek. Met behulp van een nieuwe bioreactor werd onafhankelijk gekeken naar de de effecten van pH en pO2 op de genexpressie, het metabolisme en groeifactor (VEGF) productie van geredifferentiëerde chondrocyten. Wanneer de pO2 laag wordt gehouden (5%) en de pH gevariëerd binnen een fysiologisch bereik (6.4-7.4) kon worden aangetoond dat de expressie van enkele belangrijke marker genen sterk afhankelijk is van pH. De expressies van collageen type I, SOX9 en VEGF werden significant geïnhibeerd bij lagere pH onder 5% pO2. Ook een lage pO2 had onder physiologisch neutrale pH (7.4) een inhiberend effect of genexpressie van collageen type I, terwijl HIF1a expressie lager en VEGF expressie hoger was onder lage pO2. Op eiwit niveau was het inhiberende effect van lage pH en hoge pO2 op VEGF ook aantoonbaar.

De effecten van extracellulaire pH waren niet alleen op eiwit- en genexpressie niveau duidelijk, ook het metabolisme van de cellen is erdoor beïnvloed. Onder lage pH (onder zowel hypoxische als normoxische condities) consumeerden chondrocyten minder glucose en produceerden ze minder lactaat, wat wijst op een verandering van het metabolisme (inhibitie van de oxydatieve phosphorylering en een toename van anaerobische energie productie). Het gebruik van bioreactoren om de omgeving van cellen nauwkeurig te controleren stelt ons in staat onafhankelijke effecten

van omgevingsparameters te bestuderen. Dit helpt ons niet alleen bij het beter kweken van weefsel voor regeneratieve therapieën, maar ook bij het bestuderen van de effecten in het ontstaan en de progressie van ziekten zoals OA.

Eén van die effecten, lage zuurstofspanning in redifferentiatie, wordt onderzocht in hoofdstuk 4. Chondrocyten bevinden zich in het lichaam in een omgeving van lage zuurstofspanning. Een lage pO2 wordt daarom vaak in verband gebracht met het behoud van, of differentiatie richting, het chondrocyt phenotype. Een verhoogde expressie van collageen type II onder hypoxia is hiervan het gevolg. De groeifactor TGF-β is al geruime tijd bekend als een belangrijke factor in chondrogenese. In dit hoofdstuk wordt de rol van hypoxia op de TGF-β celsignaling route in redifferentiërende chondrocyten onderzocht. Hypoxia verhoogt de expressie van de β2 isovorm van TGF, terwijl de expressie van de β1 en -3 isovormen onveranderd blijft. Tevens is de expressie van de TGF-β receptren verhoogd onder hypoxia. Chemische inhibitie van celsignaling via de TGF-β receptor onder hypoxia, of knockdown van TGF-β2 expressie met siRNA toont aan dat signalering van TGF-β2 cruciaal is voor de verhoogde COL2 expressie onder lage pO2.

Niet alleen zuurstofspanning en pH kunnen chondrocyten beïnvloeden. De toniciteit (dwz de osmotische druk) van kraakbeen heeft eveneens een belangrijke invloed op chondrocyten. De toniciteit van kraakbeen in het lichaam is hoger dan dat van plasma en van standaard kweekmedium. De effecten van een verhoogde osmolariteit op het kweekproces van chondrocyten en de onderliggende mechanismen worden onderzocht in hoofdstuk 5. Standaard kweekmedium heeft een osmolariteit van circa 280 mOsm. In dit hoofdstuk wordt aangetoond dat chondrocyten die bij een hogere, voor chondrocyten physiologische, osmolariteit (380 mOsm) gekweekt worden een hogere expressie van de chondrogene marker COL2 behouden, terwijl de expressie van de dedifferentiatiemarker COL1 onderdrukt wordt, vergeleken met kweken bij 280 mOsm. De proliferatiesnelheid wordt echter niet beïnvloed door de hogere osmolariteit, wat duidt op een beter behoud van het chondrocyt phenotype onder 380 mOsm. Nog hogere osmolariteit is daarentegen slecht voor

de chondrocyten; proliferatie onder 480 mOsm is sterk vertraagd en 580mOsm leidt tot celdood. De transcriptie factor NFAT-5 (nuclear factor of activated T-cells 5) blijkt een cruciale rol te spelen in dit proces. Inhibitie van NFAT-5 met behulp van siRNA onderdrukt de inductie van COL2 expressie onder 380 mOsm, terwijl COL1 expressie verhoogd was ten opzichte van de controle condities. Deze resultaten tonen aan dat ook osmolariteit een krachtige parameter is die het chondrocyt phenotype beïnvloedt in kweek.

Het gebruik van stamcellen voor de regeneratie van weefsels biedt een interessant alternatief voor het gebruik van lichaamseigen cellen uit het aangedane weefsel. Mesenchymale stamcellen (MSCs) zijn in staat te differentiëren naar bot, vet en kraakbeen en zijn daarom uitermate veelbelovend als alternatief voor kraakbeen regeneratie. Hoofdstuk 6 reviewt daarom de effecten van hypoxia op deze cellen. Hoewel er nog een gebrek aan consensus is over de exacte effecten van hypoxia op MSCs (als gevolg van het grote verschil in experimentele opzetten) lijken deze cellen goed in staat zich aan te passen aan een omgeving met lage zuurstofspanning. Ze zijn en staat te overleven onder lage zuurstofspanning en indien de zuurstofspanning niet te laag wordt lijkt het zelfs proliferatie te stimuleren. Hypoxia kan in sommige gevallen ook direct de differentitie van cellen beïnvloeden, de onderliggende mechanismen hiervan zijn echter nog niet bekend. Tenslotte heeft zuurstofspanning ook nog effect op de paracriene effecten van MSCs en op het recruteren van MSCs in de bloedbaan. Het gebruik van hypoxia om celgedrag te beïnvloed, in plaats van te vertrouwen op groeifactoren, medicijnen of genetische manipulatie, biedt interessante mogelijkheden voor onderzoekers die de mogelijkheden van regeneratieve geneeskunde sneller naar de kliniek willen brengen.

In dit proefschrift onderzochten we verschillende omgevingsfactoren; mechanische belasting, zuurstofspanning, pH en toniciteit, en hun rol in celkweek voor kraakbeenvervangende therapieën. Het is duidelijk dat deze factoren een sterke rol spelen in het celkweekproces. Een goede controle van deze factoren gedurende dit proces, bijvoorbeeld door gebruik van bioreactoren, zal ons in staat stellen om de uitkomst van het kweekproces

beter te beheersen. Eveneens kan deze controle gebruikt worden om de pathogenese van multi-factoriële aandoening zoals osteoarthrose te onderzoeken.

9

Chapter 9

English summary

Articular cartilage is a smooth, white tissue that is responsible for the smooth and frictionless movement of joints. It also functions as a shock absorber that distributes load evenly over the underlying bones.

Articular cartilage consists of relatively few main components. Glycosaminoglycans are negatively charged and therefore attract a lot of water, which causes the tissue to swell. As a result, the main component of cartilage is water. This swelling is mainly counteracted by the presence of (type II) collagen fibers. In addition, the integrity of the tissue is maintained by only one cell type, the chondrocyte. Despite the fact that articular cartilage has few main constituents, it is an incredibly complex tissue that is not able to repair itself correctly. Consequently it is a tissue that has received a lot of attention in the field of tissue engineering, which uses a combination of cells, (bio)materials and growth factors to produce ex-vivo generated tissues.

There are many ways to influence cell growth in a culture environment. One of those ways is by directly altering this environment. This can be done with the aid of bioreactors. For example, bioreactors are able to mechanically load cells, or precisely control the oxygen tension and pH of the culture environment. Through the use of these manipulations, cells can be affected in such a way as to obtain better culture results.

In vivo cartilage is constantly loaded due to daily movements. In healthy cartilage this loading helps to maintain the integrity of the tissue; chondrocytes respond to the load by synthesizing structural proteins such as proteoglycans and type II collagen (COL2). Chondrocytes from osteoarthritic cartilage, or chondrocytes that have been cultured in vitro, respond differently to this loading. In chapter 2 of this thesis we examined the results of loading on chondrocytes in different stages of (de)differentiation on gene expression level. When chondrocytes from osteoarthritic cartilage are cultured and then loaded, the expression of the structural proteins, AGCN and COL2, is lowered, while the expression of matrix degrading enzymes, MMP-1 and -3, is upregulated. In contrast, primary chondrocytes show a more anabolic response to loading, including upregulation of AGCN and COL2. Partial redifferentiation

causes a reduction in the downregulation of the gene expression of AGCN and COL2 in response to loading. These results have implications for the expansion procedures that are used in autologous chondrocyte transplantation and for the design of treatment plans of patients treated with cultured chondrocytes.

Articular cartilage does not contain any blood vessels for the supply of oxygen and the removal of waste products. Chondrocytes therefore exist in an environment where oxygen tension (pO2) and pH are lower than normal. In chapter 3 the effect of these environmental parameters on chondrocytes in redifferentiation culture is examined. With the aid of a novel bioreactor system we looked at the independent effects of oxygen tension and pH on gene expression, metabolism and growth factor (VEGF) production of redifferentiated chondrocytes. When the pO2 was maintained at 5% and the pH was varied over a physiological range (6.4-7.4) it was shown that the expression of several important marker genes varies strongly with extracellular pH. The expression of collagen type I, SOX9 and VEGF were strongly inhibited by low pH at 5% pO2. A low pO2 also has an inhibiting effect on the gene expression of COL1 at neutral pH (7.4), while the expression of HIF-1a and VEGF is upregulated under low pO2.

The inhibiting effect of low pH and high pO2 was also demonstrated on protein level through ELISA. The effects of the extracellular pH on the cells are not only apparent on gene and protein level. It also affects the cell's metabolism. At low pH (at both hypoxic and normoxic condition) cells consumed less glucose and produced more lactate, which indicates an inhibition of the metabolism. The use of bioreactors to accurately control the culture environment of cells allows us to more accurately study the independent effects of various environmental parameters. This will benefit not only our cell culture efforts for regenerative therapies, but also enables studying these parameters in the pathogenesis of progressive diseases such as OA.
One of these parameters, low oxygen tension in redifferentiation, is further examined in chapter 4. In vivo chondrocytes exist in an environment of low oxygen tension. A low pO2 is therefore often associated with a

maintenance of, or differentiation towards, the chondrocyte phenotype. An elevated expression of collagen type II is the result. The growth factor TGF-β has long been known for its important role in chondrogenesis. In this chapter the role of hypoxia on TGF-β cell signaling is examined in redifferentiating chondrocytes. Hypoxia upregulates the expression of the β2 isoform of TGF, while the expression of the β1 and β3 isoforms remains unaltered. Expression of the receptors for TGF-β is also upregulated under hypoxia. Chemical inhibition of TGF-β receptor mediated cell signaling, or knockdown of TGF-β2 using siRNA, shows that signaling via TGF-β2 is crucial for elevated COL2 expression under low pO2.

Not only oxygen tension and pH affect chondrocytes. The tonicity (i.e. osmotic pressure) in cartilage also has a profound effect on chondrocytes. The tonicity of cartilage is higher than the tonicity of plasma and standard culture medium. The effects of an elevated osmolarity on the culture process of chondrocytes and the underlying mechanisms are examined in chapter 5. Standard culture medium has an osmolarity of 280 mOsm. In this chapter it is shown that chondrocytes that are cultured at a higher, for chondrocytes physiological, osmolarity (380 mOsm) retain a higher expression of the chondrocyte marker COL2, while expression of the dedifferentiation marker COL1 is suppressed, compared to culture at 280 mOsm. The rate of proliferation is not affected by the difference in osmolarity, which indicates a better retention of the chondrocyte phenotype at 380 mOsm. Even higher osmolarities inhibit proliferation (i.e. at 480 mOsm) and 580 mOsm causes cell death. The transcription factor NFAT-5 (nuclear factor of activated T-cells) plays a crucial role in this process, as inhibition of NFAT-5 using siRNA supresses the induction of COL2 expression under 380 mOsm, while COL1 expression is upregulated compared to control conditions. These results show that osmolarity is another potent parameter that influences the chondrocyte phenotype in culture.

The use of stem cells for tissue regeneration offers an interesting alternative for the use of autologous chondrocytes. Mesenchymal stem cells (MSCs) have the ability to differentiate towards bone, fat and cartilage and are therefore especially promising as an alternative for cartilage regeneration.

Chapter 6 reviews the effects of hypoxia on these cells. Although there remains a lack of consensus on the effects of hypoxia on MSCs (due to the large variation in experimental procedures) it appears clear that these cells are well able to adapt to hypoxia. They can survive low oxygen tensions for prolonged time periods and, if the oxygen tension does not drop too low, it even appears to support their proliferation. In some cases hypoxia directly affected MSC differentiation, although the underlying mechanisms have yet to be elucidated. Finally, hypoxia also affects the paracrine activity of MSCs and their recruitment to the site of injury. The ability to culture cells and enhance their rate of proliferation, differentiation potential, and in-vivo survival simply by lowering oxygen tension offers exciting new potential for tissue engineering. The possibility of positively altering cell behavior on this scale without the use of additional growth factors, drugs or genetic manipulation should attract the interest of many researchers aiming to bring regenerative medicine closer to the clinic.

In this thesis we investigated various environmental parameters; mechanical loading, oxygen tension, pH and tonicity and their roles in cell culture for cell-based cartilage replacement therapies. It is clear that these parameters have a strong effect on the entire culture process. Strict control of these parameters during culture, through the use of bioreactors, will allow us to better control the outcome of the cell culture process and as a result improve the effectiveness of cell-based therapies in cartilage replacement. Additionally, the strict and independent control can be employed to study the pathogenesis of multi-factorial diseases such as osteoarthritis.

Abbreviations

ACI	Autologous chondrocyte implantation
ACAN	Aggrecan
ATP	Adinosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
cDNA	Complementary DNA
COL1	Collagen type I
COL2	Collagen type II
COL10	Collagen type X
CS	Chondroitin sulfate
CX3XR1	CX3C chemokine receptor
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO/EPOR	Erythropoietin/ Erythropoietin receptor
ERK	Extracellular signal-regulated kinase
FCD	Fixed charge density
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gly	Glycine
HA	Hyaluronic acid
HAS	Hyaluronan synthase

HB-EGF	Heparin-binding EGF-like growth factor
HIF	Hypoxia inducible factor
HUVEC	Human umbilical vein endothelial cell
IFN-γ	Interferon-γ
IGF	Insulin-like growth factor
IL	Interleukin
KS	Keratan sulfate
MACI	Matrix-induced autologous chondrocyte implantation
MCP	Methyl-accepting chemotaxis protein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
OA	Osteoarthritis
Oct-4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PID	Proportional-integrative-derivative
pO_2	Partial oxygen pressure
PRG-4	Proteoglycan-4/superficial zone protein
p38-MAPK	p38-Mitogen activated protein kinase
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDF-1α	Stromal-cell derived factor 1α
SOX9	SRY (sex determining region Y)-box 9
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Curriculum Vitae

Ruud Das werd geboren in Valkenswaard (2 juni 1980), waar hij in 1998 aan het Were Di college zijn gymnasium diploma behaalde. Plannen voor een studie geneeskunde werden in de kiem gesmoord door de numurus fixus, maar gelukkig was daar ook de nieuwe studie BioMedische Technologie aan de Technische Universiteit Eindhoven. Ondanks de mogelijkheid om een jaar later alsnog met geneeskunde te beginnen, bleef hij bij de keuze voor biomedische technologie, vanwege de unieke combinatie van het medische en technische. Tijdens een buitenlandse stage in Parijs, bij Prof. Christian Oddou, werd de basis voorzijn interesse in celkweek met bioreactoren gelegd. Na een kort intermezzo voor een master scriptie bij Prof. Rik Huiskes en dr. René van Donkelaar, waarin met een computermodel gekeken werd naar reorganisatie van collageenvezels in articulair kraakbeen, begon hij in 2005 aan zijn promotie aan de Erasmus Universiteit in Rotterdam. In dit onderzoek, onder leiding van promotor Prof. Harrie Weinans, stond celkweek met bioreactoren wederom centraal. Deze trend werd doorgezet, en sinds 2010 is hij werkzaam als (senior) scientist bij Xpand Biotechnology, waar bioreactor technologie wordt ontwikkeld voor het kweken van cellen voor toepassingen in de celtherapie.

Dankwoord

Het schrijven van een proefschrift is een enorm solitaire aangelegenheid. Daarom is het des te belangrijker dat alle jaren voorafgaand aan die laatste keer "save as" gevuld zijn met mensen die er voor zorgen dat je de moed erin houdt. Of dat nu is door wetenschappelijke stimulatie, de broodnodige afleiding na (en soms tijdens) werk, of simpelweg het aanhoren van een stortvloed aan geklaag als het hart weer eens gelucht moet worden, iedereen heeft eraan bijgedragen dit boekje er uiteindelijk ligt. Ergens hoop ik dat deze dankbetuiging slechts een summiere bevestiging wordt van wat iedereen al weet, maar eigenlijk kan je nooit te ver gaan in het uitspreken van waardering, toch?

Professor Weinans, beste Harrie. Bedankt dat je me de mogelijkheid hebt gegeven me wetenschappelijk te ontplooien. De vrijheid die je promovendi geeft om een eigen richting te vinden heb ik zeker altijd gewaardeerd. Ondanks dat dat, gekoppeld aan mijn inherente kritisch-pessimistische blik, niet altijd ideaal uitpakte, heeft het me gevormd tot de wetenschapper die ik nu ben. En uiteindelijk ben ik dan toch die sprinter die ook een marathon heeft volbracht.

Dr. Jahr, beste Holger. Bedankt voor alle enthousiaste steun door de jaren heen. Ik was altijd onder de indruk van de eindeloze stroom ideeën die van jouw kant af kwamen en van je bijna encyclopedische moleculair biologische kennis. Je bent er in geslaagd om deze technicus in ieder geval een hoop biologische handvatten mee te geven voor de rest van mijn carrière. Bovendien was het enorm verhelderend om telkens weer jouw visie te horen over hoe we een studie naar een hoger plan konden tillen.

Professor van Osch, beste Gerjo. Ondanks dat je slechts kort betrokken was bij de begeleiding van mijn promotie, is je input altijd enorm waardevol geweest. Ook naderhand nog. Jouw positieve kijk was steeds weer enorm opbeurend en ik ben daarom ook blij dat onze paden elkaar nog steeds kruisen.

Uiteraard kan ik alle collega's niet vergeten. Zij zijn het immers die me altijd de dag door hielpen. Yvonne, YBJ, ik mis de legio koffie breaks, feestje, drankjes die we deelden. Wendy, Crowbar, bedankt voor alle gesprekken (soms leuk, soms serieus, altijd welkom), vooral bij de LAF-kast onder het genot van Radio 2. Nicole, Chuckles, jouw vrolijkheid was altijd een boost op lange dagen. Anna van der Windt-signaling, onze samenwerking was helaas maar van korte duur, want ik denk dat er nog veel meer moois in had gezeten. Hopelijk zien we elkaar bij de volgende marathon. Esther, E*, jouw hulp in het laatste deel van mijn onderzoek heeft me enorm veel geholpen. Gerben, Gerbil, het was fantastisch om met je door New Orleans en Ierland te toeren en natuurlijk alle borrels te doen. Yvonne (ik vrees alleen voor mij Yvonne nummer 1), het was geweldig om de oude BMT-garde tegenover me te hebben. Hopelijk blijven we elkaar tegen komen. Erwin, hoewel we elkaar relatief misschien maar weinig gesproken hebben heb ik je rust en bereidheid tot helpen altijd erg gewaardeerd. Olav en Tom, misschien niet vreemd dat ik jullie in één adem noem, vrijwel onze hele promotie periode samen meegemaakt en dankzij jullie ook weer net dat beetje makkelijker. Miriam, jij mag in dit rijtje natuurlijk zeker niet ontbreken. Je was maar kort op ons lab, maar ik ben heel blij dat we na al die tijd nog steeds contact houden. En verder wil ik iedereen die het lab orthopedie bevolken, of hebben bevolkt, bedanken. Sandra, Marieke, Katja, Daan, Robert-Jan, Stefan, Elian, Justus, Marloes, Mieke, Jasper en alle anderen die ik onverhoopt over heb geslagen, bedankt voor alle steun, groot of klein. Een speciale vermelding is ook nog op z'n plaats voor Martijn en Marco. Martijn, bedankt voor alle keren dat ik weer bij je terecht kon als er weer gesleuteld moest worden aan de bioreactor. Marco, thanks for putting a brother up. Geweldig dat ik een maand kon overbruggen in Rotterdam dankzij jou.

Eric, I don’t think anyone is surprised that you’re there as my paranimf. I remember Gerjo telling we’d get a guest from Ireland and that we should make him feel at home. I do believe I can say mission accomplished. I am really happy that our epic time in Rotterdam now gets a continuation in Utrecht. Looking forward to having you and Aleisha over a lot!
De academische wereld is een small world after all, vandaar dat ik gelukkig ook kon rekenen op vrienden die daar buiten staan. Of dan toch in ieder

geval buiten de life science tak. Geert, als er iemand is die way back gaat, dan ben jij het wel. Wat goed dat we nu eigenlijk weer praktisch buren zijn! Laurens en Alwies, ook jullie twee noem ik een adem. Als laatste van ons drie heb ik dit boekje af, maar dan wel mede door de steun die ik altijd heb gehad van jullie. Ik ben blij dat we nu nog steeds bij elkaar over de vloer komen.En uiteraard de hele garde uit Valkenswaard. Hoewel het contact de laatste tijd minder intensief is, zijn jullie er op de momenten die tellen. Hans, Will, Willem, Floris (en aanhang :P), bedankt! Maar de grootste escapistische bezigheid was toch wel spelen in mijn bands. Remy, als common denominator, cool dat we zo lang deze passie hebben kunnen delen. Verder alle oud-bandleden (Laurens (alweer), Koen, Bouke, Maarten en Rob), het was geweldig om de oefenruimte en het podium met jullie te delen. Tenslotte moet ik hier ook zeker nog mijn collega's van het Smulhuis vermelden. Jullie hebben te transitie naar Utrecht enorm makkelijk gemaakt.

Sweet Jinyi, the last year has been amazing! I hope we can have many more of those together. My life is better if we can be idiotic together. Never a dull moment!!

De laatste paragrafen zijn gereserveerd voor de belangrijkste mensen in mijn leven, mijn familie. Joost, maar anderhalf jaar zit er tussen ons, en dat is te merken. Ik kan me geen tijd herinneren dat we niet dingen samen deden, en zelfs vandaag met jou als paranifm naast me. Ik hoop dat we dat ook nog heel lang blijven doen. Ik ben er trots op dat je alles op alles zet om je droom te verwezenlijken en ik ben er van overtuigd dat het gaat lukken.

Hetty, ik durf vol overtuiging te zeggen dat je de beste zus ooit bent. Je koos voor een heel andere aanpak, maar zie maar eens waar je nu bent! Ik zal als trotse oom nu veel vaker bij jou, Jorden, Evi en Iza op bezoek komen.

Tante Truus, ik ben maar wat blij dat je zo close was met ons mam, en dat we daardoor altijd zo nauw contact hebben gehad. Geweldig dat jij en ome Harrie er altijd zijn (geweest) voor ons.

Pap, ik hoop dat wat ik wat ik hier schrijf niet als een verrassing komt. Ik hoop dat je al lang weet dat ik dit alles niet had kunnen doen zonder de steun die ik altijd van jullie kreeg. Ik hoop ook dat je weet dat ik trots ben op de manier waarop je in het leven staat. Toen al, maar vooral nu. Bedankt, voor alles.

Mam, nog steeds onwerkelijk dat de belangrijkste persoon uit deze hele lijst degene is die het niet zal lezen. Jouw steun en toewijding, niet alleen aan je gezin, maar ook ver daarbuiten, is voor mij een inspiratie geweest. Dit boekje, en al het andere dat ik doe, is dan ook voor een groot deel jouw verdienste.

PhD portfolio

Courses

Classical Methods for data-analysis, NIHES, 2005
Cell-based therapies and tissue engineering, Case Western Reserve University, Cleveland OH (USA), 2008

Conferences

Oral presentations
Das, R; Kreukniet, M; Jahr, H; Van Osch, G J; Weinans, H: Regulation of oxygen tension and pH in a bioreactor for cartilage tissue engineering; DPTE 2007: Noorwijkerhout

Das, R; Kreukniet, M; Jahr, H; Van Osch, G J; Weinans, H: Regulation of oxygen tension and pH in a bioreactor for cartilage tissue engineering; TERMIS 4-7 september 2007: London, UK

Das, Ruud; Jahr, Holger; Kreukniet, Martijn; Oostra, Jaap; van Osch, Gerjo; Weinans, Harrie: Control of oxygen tension and pH in a bioreactor for cartilage tissue engineering; 4ème Colloque International en Bioingénerie et Biothérapies 26-28 september 2007: Nancy, Frankrijk.

R.H.J. Das, H. Jahr, M. Kreukniet, J. Oostra, G.J.V.M. van Osch, H. Weinans: A Bioreactor for Control of Oxygen Tension and pH in Cartilage Tissue Engineering; NBTE 12-13 december 2007 Lunteren.

Das R, Weinans H, Jahr H: Hypoxia-mediated chondrogenic marker expression: implications for cartilage tissue engineering; NBTE 15-16 Dec 2008: Lunteren.

R.H.J. Das, H. Weinans, H. Jahr: Hypoxia induced chondrocyte redifferentiation through endogenous TGF-β / ALK5 signaling. ORS 6-9 Mar 2010, New Orleans, USA

Poster presentations

Das R, Jahr H, Verhaar J, van Osch G, Weinans H. Effects of deformation on primary and culture-expanded chondrocytes. Tissue Engineering and Regenerative Medicine International Society Europe. Rotterdam, The Netherlands. 8-11 October 2006

Das, R H J; Jahr, H; Van Osch, G J V M; Verhaar, J A N; Weinans, H: Deformation is detrimental to cartilage matric formation by expanded osteoarthritic chondrocytes. ORS 11-14 februari 2007 San Diego, USA.

R.H. Das, H. Jahr, J. Verhaar, G. van Osch, H. Weinans: Deformation is detrimental to cartilage matrix formation by expanded chondrocytes; ICRS 29 september – 2 oktober 2007 Warsaw, Polen.

R.H.J. Das, J. Malda, G. van Osch, H. Weinans, H. Jahr: Substrate geometry and chondrocyte phenotype; ICRS 29 september – 2 oktober 2007 Warsaw, Polen.

Das R, Weinans H, Jahr H: Hypoxia in cartilage tissue engineering: control through TGF-β? DPTE 11-12 Nov 2008: Noordwijkerhout, NL.

R.H.J. Das, H. Jahr, M. Kreukniet, J. Oostra, G.J.V.M. van Osch, H. Weinans: Control of pH and Oxygen Tension in a Cartilage Tissue Engineering Bioreactor System. ORS 22-25 Feb 2009, Las Vegas, USA

Teaching activities

Supervising M.Sc. internship Daniel Venegas Suarez
Supervising M.Sc. internship Eefje Bierhof
Supervising M.Sc. internship Anne Sofie Jacobsen

List of publications

Das RH, Jahr H, Verhaar JA, van der Linden JC, van Osch GJ, Weinans H. In vitro expansion affects the response of chondrocytes to mechanical stimulation. Osteoarthritis Cartilage. 2008 Mar;16(3):385-91.

Das R, Kreukniet M, Oostra J, van Osch G, Weinans H, Jahr H. Control of oxygen tension and pH in a bioreactor for cartilage tissue engineering. Biomed Mater Eng. 2008;18(4-5):279-82.

Das RH, van Osch GJ, Kreukniet M, Oostra J, Weinans H, Jahr H: Effects of individual control of pH and hypoxia in chondrocyte culture, J Orthop Res, 2009 Oct 7;28(4):537-545, PMID: 19813243

Das R, Jahr H, van Osch GJ, Farrell E., The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng Part B Rev. 2010 Apr;16(2):159-68

Kempen DHR, Custers RJH, Delawi D, Geuze RE, Rutgers JPHJ, Fedorovich NE, Rutgers M, Das RHJ, Jansen JHW, Piscaer TM, Sniekers YH. Ontwikkelingen binnen het orthopedisch onderzoek - Verslag van de 53e jaarvergadering van de Orthopedic Research Society, Nederlands Tijdschrift voor Orthopaedie, 2007 juli, vol 14: 74-76

van der Windt AE, Haak E, Das RHJ, Kops N, Welting TJM, Caron MMJ, van Til NP, Verhaar JAN, Weinans HH, Jahr H, Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro, Arthritis Research & Therapy 12/3, p1-27 (2010)