# Chapter 3
# Targeting Inflammatory Processes for Optimization of Cartilage Homeostasis and Repair Techniques

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**Abstract** The outcome of cartilage repair techniques is often hampered by unwanted ossification (e.g. intralesional osteophytes) at the site of the repaired cartilage. Furthermore, stimulating progenitor cells towards chondrocytes and locking them in their desired state is another important hinge point in cartilage repair techniques. Studying the cartilage formation process by endochondral ossification may provide important clues which further enhance cartilage repair techniques in general and may provide crucial information to prevent unwanted ossification in particular. During endochondral ossification mesenchymal progenitors differentiate into proliferative chondrocytes which gradually further differentiate into hypertrophic chondrocytes and finally die by apoptosis; the remaining scaffold is mineralised towards bone. This process takes place in growth plates, during fracture healing and in part during development of articular cartilage, where the endochondral ossification halts at the chondrogenic phase. While inflammation is generally regarded as a negative factor for joint homeostasis and cartilage development, it is also known that inflammation is the first and essential phase of tissue repair in general and bone fracture healing via endochondral ossifcation indeed also depends on haematoma formation and subsequent inflammatory microenvironment. Recently, a growing body of experimental evidence has been published, showing that inflammatory molecules (e.g. NF-κB, COX-2, iNOS, TNFα,

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P.J. Emans, L. Peterson (eds.), *Developing Insights in Cartilage Repair*,
DOI 10.1007/978-1-4471-5385-6_3, © Springer-Verlag London 2014

interleukins) and their down-stream pathways are not only associated with cartilage degeneration, but are also crucially involved in the initiation of the chondrogenic differentiation process and regulation of cartilage hypertrophy and mineralization. The data described in these reports suggest that one could use these inflammatory pathways for cartilage regenerative medicine, as the initiation of chondrogenic differentiation is a crucial moment for progenitor cell-based cartilage repair techniques. Furthermore, targeting inflammatory mediators may also provide a potential pharmacological approach to prevent or decrease chondrocyte hypertrophic differentiation and subsequent bone formation (e.g. intralesional osteophytes) in cartilage repair techniques.

This chapter describes important characteristics of hyaline articular cartilage, drawbacks of current cartilage repair techniques, the process of endochondral ossification and how inflammation related molecules are involved in different phases of endochondral ossification. In addition, this chapter discusses how better insight into these pathways may provide novel molecular tools to modulate chondrogenesis in cartilage regenerative medicine.

**Keywords** Cartilage repair • Intralesional osteophyte • Inflammation • Chondrogenesis • Progenitor cells • NF-κB • COX-2

**Key Points**

- The outcome of cartilage repair techniques is often hampered by unwanted ossification (e.g. intralesional osteophytes) at the site of the repaired cartilage.
- Studying the cartilage formation process by endochondral ossification may provide important clues which further enhance cartilage repair techniques in general and may provide crucial information to prevent unwanted ossification in particular.
- While inflammation is generally seen as a negative factor for joint homeostasis and cartilage development, it is also known that inflammation is the first and essential phase of tissue repair in general.
- One may implement these inflammatory pathways for cartilage regenerative medicine, as the initiation of chondrogenic differentiation is a crucial moment for progenitor cell-based cartilage repair techniques.

## 3.1 Introduction: Cartilage

Motion in articular joints is possible by a truly remarkable material both structurally and functionally, named hyaline articular cartilage [1–4]. This articular cartilage is able to withstand an enormous amount of intensive and repetitive forces combined with low friction and thereby allows easy movement. The extracellular matrix (ECM) of cartilage determines these cartilage-specific functions and is mainly

composed of water (65–80 %), collagens (12–21 %), proteoglycans(6–10 %) and other glycoproteins (2–3, 5 %) [5]. Only 1–5% of the articular cartilage volume consists of chondrocytes, the main cell type found in articular cartilage [6]. Furthermore, cartilage is characterized by the absence of blood vessels, lymphatics and nerve fibers. This implicates that cartilage is mainly hypoxic and chondrocytes have to receive their nutrients and oxygen via diffusion from the synovial fluid, through the surrounding extracellular matrix and from the underlying subchondral bone [7]. Cartilage defects can arise due to trauma or cartilage degeneration, but are generally difficult to diagnose [8, 9]. Since cartilage has no nerve fibres, cartilage lesions often present with only (minor) effusion of the affected joint or without symptoms at all. Symptoms as joint pain, locking phenomena and reduced or disturbed joint-function may arise from other tissues or structures likely to be damaged upon trauma (e.g. subchondral bone, ligaments or menisci). Although progenitor cells are found in the superficial layer of articular cartilage [10, 11], cartilage has a limited ability for self-repair [12, 13]. This was already recognized in 1743 when the British surgeon William Hunter made the now famous statement: "*From Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that once destroyed it is not repaired*" [14]. This observation is one of the main reasons for clinicians and researchers to explore ways for cartilage repair. Because, when left untreated, the joint surface will deteriorate even further, ultimately leading to osteoarthritis (OA).

## 3.2 Calcification in Cartilage Repair Techniques

Cartilage restoration implies methods to heal or regenerate the joint surface, with or without the subchondral bone, into healthy hyaline articular cartilage to restore joint functioning. To date there are multiple fruitful cartilage repair techniques; however, the ultimate cartilage repair technique has not been found yet. One of the main drawbacks is unwanted ossification (and formation of intralesional osteophytes) at the site of the repaired cartilage [15, 16].

As described above, the properties of the (hyaline) cartilage matrix are essential to withstand the repetitive compressive forces which are put on the joints, allowing easy movement. Hypertrophic cartilage or even mineralized cartilage in the articular surface has inferior properties concerning resisting repetitive mechanical loading to that of hyaline cartilage and will thereby result in the further destruction of the joint cartilage and can act as a source of pain [17]. Chondrocyte hypertrophic differentiation is thus of concern in cartilage repair techniques but also in the onset of osteoarthritis, as e.g. markers for hypertrophic differentiation are specifically expressed at early stages of OA [18–20]. In addition to formation of hypertrophic cartilage, stimulating progenitor cells towards extracellular matrix-producing chondrocytes and keeping them in their desired differentiation state is another important factor to consider in cartilage repair techniques [15, 16].

Bone marrow stimulating techniques such as microfracture, abrasion and subchondral drilling are easy applicable, cheap and reliable methods to attempt

the functional repair of cartilage defects. These techniques are based on the penetration of the subchondral bone allowing ingress of bone marrow stem cells into the site of the damaged cartilage [21–31]. These cells are thought to differentiate into the chondrogenic lineage and become functional ECM-producing chondrocytes which replace the damaged cartilage. However, formation of fibrocartilage and calcification of repaired tissue hampers clinical outcome on the long term [16, 30]. Another source of chondro-progenitor cells can be found in the cambium layer of the periosteum and in the perichondrium. These cells have been described to have a chondrogenic potential as well [12, 32–41]. Covering cartilage defects with periosteum-derived grafts (periosteal arthroplasty) is therefore an explored strategy to treat cartilage defects [42–50]. On short term, results were found to be quite promising in giving initial cartilage repair [43–46, 48]. Unfortunately, on the long term results were poor and failure was related to overgrowth and calcification of the graft [42].

Other techniques imply the transplantation of adult chondrocytes or cartilage such as mosaicplasty (Osteochondral Autograft Transfer System; OATS), allografts and Autologous Chondrocyte Transplantation (ACT), which may overcome these drawbacks. Mosaicplasty or OATS involves harvesting osteochondral plugs from a relatively less weight-bearing region of the joint and subsequent implantation of these plugs into the articular defect [51–54]. The use of allografts can overcome possible donor site morbidity [52, 53, 55–61] or shortage of graft material. ACT refers to a cell- based cartilage repair procedure, where cartilage is harvested arthroscopically from a less weight-bearing region of the joint and transferred to a specialized laboratory where the chondrocytes are enzymatically released from their matrix and expanded *in vitro*. The patient then undergoes a second operation where the *in vitro* expanded chondrocytes are re-implanted at the damaged site of the articular cartilage, in combination with a covering membrane (periosteum of biomembrane) [62–64] or pre-seeded in a matrix (Matrix Assisted Chondrocyte Transplantation; MACT) [65]. Nevertheless, the use of these techniques is restricted due to a limited availability of autologous cartilage (mosaicplasty) or donors, possible disease transfer (allografts), or expensive and time consuming logistics and culture methods (ACT). Furthermore, cartilage hypertrophy is also seen after ACT, albeit more in the periosteum-covered ACT than in de matrix-assisted ACT [66, 67].

The use of progenitor cells for cartilage repair remains of interest. When applied for cartilage repair, stem cells have a natural tendency to differentiate into the chondrogenic lineage, via a process called endochondral ossification, forming cartilaginous tissue in the damaged area which gives initial cartilage repair. However, on the long term, progenitor-based grafts tend to calcify as a natural result of the endochondral ossification process. Microfracture and periosteum or perichondrium plasty [42, 68], are good examples here of, all showing adverse ossification and/or formation of interlesional osteophytes. Recently these osteophytes have also been described when articular cartilage was transplanted into a defect [69].

Beside appropriate induction of differentiation, maintaining these progenitor cells in the desired differentiation state and preventing them from further

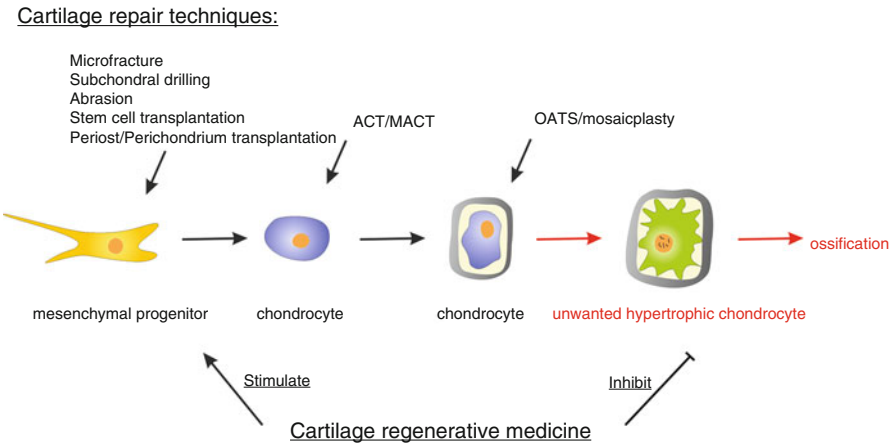


**Fig. 3.1** Different phases of chondrogenic differentiation and targets of cartilage regenerative techniques

hypertrophic differentiation is therefore a major challenge for stemcell-based cartilage repair strategies [15]. Studying the process of endochondral ossification and further unraveling how and why articular chondrocytes maintain their phenotype and are saved from hypertrophy may enhance cartilage repair techniques by generating stable cartilage. A suggestion in which stage during the endochondral process the different cartilage repair techniques are positioned is given in Fig. 3.1.

## 3.3 Chondrogenic Phase of Endochondral Ossification

Chondrogenic differentiation encompasses the commitment and differentiation of chondro-progenitor cells towards chondrocytes (see Fig. 3.1). *In vivo*, chondrogenic differentiation is almost exclusively initiated from local mesenchymal progenitor cells that reside in cartilaginous tissue (growth plate resting zone or the articular cartilage superficial layer [70, 71]) or in surrounding fibrous tissues (e.g. periosteum [37, 72]). *Ex vivo* (*in vitro*), however, chrondrogenic differentiation has been reported from various primary (mesenchymal) progenitor cell sources including synovial fluid/membrane, adipose tissue, induced pluripotent stem cells (iPS [73]), bone marrow and many more [74].

In addition to providing articulating joint surfaces with functional cartilage and maintaining cartilage integrity, chondrogenic differentiation also plays an essential role during endochondral ossification (Fig. 3.1). Endochondral ossification underlies skeletogenesis and bone fracture healing and is a developmental process during which cartilaginous primordia are gradually replaced by bone tissue. Growth plate chondrocytes originating from the resting zone or fracture callus chondrocytes originating from mesenchymal progenitors gradually proliferate, produce a

| Differentiation step | Extracellular martrix markers | Regulatory markers | Growth and differentiation factors |
|---|---|---|---|
| Chondrogenic progenitor cells (mesenchymal cells) | Col1a1 | Sox9, Runx2 | Shh, TGF-β |
| Prechondrocytes | Ncam1, Tnc | Sox9, L-Sox5, Sox6 | TGF-β FGF-2, BMP-2,4,7 Wnt, PTHrP |
| Early chondrocytes | Col2a1, Acan, Crtl1 | Sox9, L-Sox5, Sox6 Nkx3.2, Atf2, Creb, Fgfr3 | TGF-β FGF-2, BMP-2,4,7 Wnt, PTHrP |
| Chondrocytes (columnar) | Col2a1,Col9a1, Col11a1, Acan, Crtl1, Comp, Matnl GAGs | Sox9, L-Sox5, Sox6 Nkx3.2, Atf2, Creb, Fgfr3 | TGF-β FGF-2, BMP-2,4,7, IGF-1 Wnt, PTHrP |
| Prehypertrophic chondrocytes | Col2a1,Col9a1, Col11a1, Col10a1 Acan, Crtl1, Comp, Matnl GAGs | Runx2, Runx3, Ihh, Pthr1 | Wnt/β-catenin, BMP-2,7, TGF-β |
| Hypertrophic chondrocytes | Co110a1 | Runx2, Runx3, Mef2c | VEGF, Wnt/β-catenin, BMP-2,7 |
| Terminal chondrocytes | MMP13, Alp, Opn | Runx2, c-Maf | VEGF, Wnt/β-catenin |

**Fig. 3.2** Markers for chondrogenic differentiation. Schematic representation of successive steps of chondrogenic differentiation during endochondral ossification with schematic representation of the cells, major extracellular matrix markers, regulatory markers and growth and differentiation factors expressed at each step

cartilaginous matrix and further differentiate into mineralized hypertrophic chondrocytes which finally die by apoptosis. The remaining mineralized extracellular matrix provides a molecular scaffold for infiltrating osteoblasts and osteoclasts to adhere to and remodel, setting the stage for *de novo* bone deposition [75, 76].

Notably, chondrocytes in articular cartilage retain their chondrocyte phenotype and, except for chondrocytes near the tidemark, normally do not further differentiate into hypertrophic chondrocytes, probably due to the local microenvironment. Unfortunately, as a natural result of this endochondral ossification process, *in vitro* chondrogenic differentiation of progenitor cells for cartilage regenerative purposes tends to progress into hypertrophic differentiating chondrocytes.

### *3.3.1 Molecular Factors in Chondrogenic Differentiation*

Different phases of chondrogenic differentiation can be characterized by different functional marker molecules (Fig. 3.2). Chondrogenic differentiation starts when mesenchymal progenitor cells are triggered to differentiate into the chondrogenic lineage. Chondrogenic progenitor cells express typical ECM and cell adhesion molecules like tenascin c (Tnc), syndecan 3 (Sdc3), N-cadherin (Ncad) and Ncam1 (neural cell adhesion molecule 1). One of the first key important chondrogenic differentiation regulatory events is activation of the Sox-trio transcription factors; Sox9 (SRY-(sex determining region Y)-box9) in combination with L-Sox5 and Sox6 are responsible for commitment and differentiation in the chondrogenic lineage [77–79]. Together they drive the transcription of the important ECM genes collagen type II (Col2a1) and the main proteoglycan aggrecan (Acan) [78, 80–83]. Other ECM genes have also been shown to be under transcriptional control of Sox9,

of which collagen type IX (Col9a1), collagen type XXVII (Col27a1) and matrilin 1 (Matn1) are important ones [84–87].

Eventually the (hyaline articular) cartilage ECM consists of a collagen network which is comprised of primarily Col2a1, and additionally of Col9a1 and collagen type XI (Col11a1) which help to form and stabilize the collagen type II fibril network [88–91]. Minor quantities of Col6a1, Col12a1, Col14a1 and Col27a1 are also found in cartilage [92]. This collagen network is surrounded by a highly hydrated aggregation of proteoglycans and other glycoproteins. Glycoproteins and proteoglycans as COMP (cartilage oligomeric protein), Matrilin1 (Matn1/Crtm), perlecan (Hspg2), versican (Vcan), decorin (Dcn), biglycan (Bgn) and fibromodulin (Fmod) are characterized by their ability to interact with and support the collagen fibril network and retention and transport of growth factors [79, 93]. Aggrecan (Acan) is the main proteoglycan and forms macromolecular complexes by binding to hyaluronan via link proteins and binding of glycosaminoglycans (GAGs), such as chondroitin sulfate and keratan sulfate. The glycosaminoglycan side chains of the proteoglycans are composed of repeating disaccharide units carrying negatively charged sulphate and carboxyl groups. The resulting fixed negative charge density attracts mobile cations and water into the ECM and thus provides in the elastic properties of the tissue [94, 95]. In addition to resisting compressive forces and providing lubrication during movement, the high water retention capacity of hyaline cartilage also supports in distributing nutrients to chondrocytes. The proteoglycan aggregations, together with the quality of the collagen network determine the strength and flexibility of the cartilage tissue and ability to withstand repetitive compressive forces for which articular cartilage has been designed to [2, 4, 96, 97]. For articular chondrocytes, the differentiation process stops here and cells provide maintenance of the articular surface for life. It is important to realize that in articular cartilage the ratio of cells to ECM, and composition of the ECM are important for proper joint functioning. These are therefore factors to take into account for cartilage regenerative techniques. Based on collagen type II orientation and chondrocyte shape and distribution, four zones can be distinguished in articular cartilage [1, 3, 4]. In the superficial zone, chondrocytes are flattened and are surrounded by a thin layer of ECM, mainly composed of collagen-fibres. The fibres are oriented parallel to the articular surface and are supported by a relatively low content of proteoglycans, which results in high tensile stiffness and the ability to distribute load over the surface and protecting the deeper layers. In the transitional zone the cells and collagen fibres appear dispersed randomly [98, 99] and in this zone high concentrations of proteoglycans enable the tissue to bear compressive forces. In the deep zone, chondrocytes are grouped radially in columns and the thicker collagen fibres are arranged perpendicular to the articular surface, providing the greatest resistance to compressive forces In the calcified zone, (hypertrophic) chondrocytes are distributed sparsely and are surrounded by a calcified matrix. The calcified layer plays an integral role in securing the cartilage layer to the subchondral bone by anchoring the collagen fibrils to the subchondral bone tissue. The junction between uncalcified and calcified cartilage is called the “tidemark”. At the tidemark shear stresses are converted into compressive forces which are in turn transmitted to the subchondral bone [100].

Thus, for optimizing progenitor cell-based cartilage repair techniques it is thus of importance to not only create cells which produce enough ECM, but also that this ECM has the right composition.

### 3.3.2 Molecular Factors in Chondrocyte Hypertrophy

In contrast to articular chondrocytes, the (proliferative) chondrocytes in growth plates, or involved in fracture healing, further differentiate into hypertrophic chondrocytes which subsequently undergo a remodeling of their extracellular matrix (Fig. 3.2). These chondrocytes then exit the cell cycle and increase in cell volume up to ten times [101]. There is an increase in expression of Runx2 (Runt-related transcription factor 2) and Mef2c (Myocyte-specific enhancer factor 2C), which are important transcription factors for collagen type X (Col10a1), the main collagen found in hypertrophic chondrocytes [102–105]. Furthermore, under stimulation of Runx2 and Mef2c, hypertrophic chondrocytes also express vascular endothelial growth factor (VEGF) to stimulate vascular ingrowth [79, 106]. Also several MMPs (matrix metalloproteins) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) for breakdown of the ECM are syhthesized [79, 107]. At the final stage of hypertrophic differentiation several mineralization proteins are expressed, such as Alp (alkaline phosphatase) and osteopontin (also known as bone sialoprotein I), which mineralize the extracellular matrix [79, 106–108]. Finally, the hypertrophic chondrocytes die by apoptosis, leaving their mineralized extracellular matrix behind for osteoblasts to adhere, which will eventually remodel the matrix into bone tissue.

### 3.3.3 Growth Factors and Paracrine Regulators in Chondrogenic Differentiation

In growth plate development as well as in the development and homeostasis of articular cartilage several signaling pathways are interacting or shared between the different tissues. Indian hedgehog (Ihh) and parathyroid hormone related peptide (PTHrP) coordinate chondrocyte proliferation and differentiation in the paracrine PTHrP-Ihh feedback loop [76]. PTHrP is synthesized by proliferating chondrocytes and perichondrial cells [76] and maintains chondrocyte proliferation by activating Cyclin D1 [109] and prevents premature hypertrophy by inducing Cyclin D1-mediated degradation of Runx2 [110]. Proliferating chondrocytes located at a sufficient distance from the PTHrP source stop proliferating and become hypertrophic, Ihh synthesizing cells [111]. Ihh is expressed by prehypertrophic chondrocytes and accelerates the (hypertrophic) differentiation of proliferative chondrocytes and additionally it increases the expression of PTHrP, resulting in a feedback loop that controls the pace of chondrocyte proliferation and maturation [112–114]. Next

to the PTHrP-Ihh loop, fibroblast growth factors (FGFs) crucially regulate chondrocyte proliferation and differentiation possibly by stimulating Sox9 expression and inhibiting proliferation and Ihh expression [76]. FGF signaling is balanced by bone morphogenic protein (BMP)- signaling [115]. BMPs are described to have multiple roles during bone and cartilage formation, as well as growth plate development [116]. Interestingly; BMPs were initially discovered because of their remarkable ability to ectopically induce endochondral bone formation [117]. In a cartilage context, BMPs are involved in stimulating early chondrogenesis, cartilage maintenance and hypertrophic differentiation [116]. Especially BMP-2, BMP-4 and BMP-7 (OP-1) have been demonstrated to promote chondrogenic differentiation *in vitro* [116]. BMPs belong to the transforming growth factor beta (TGF-β) superfamily, which are important regulators of differentiation, proliferation, tissue homeostasis and -repair in general. TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) support the differentiation of mesenchymal progenitor cells into the chondrogenic lineage [118–123]. The TGF-β isoforms mainly signal through phosporylated R-Smads, which in combination with co-(transcriptional) factors regulate specific target-gene expression [124, 125]. Related to its chondrogenic properties, TGF-β signalling is also involved in the formation of osteophytes during OA [126–129]. Another important regulator of chondrogenic differentiation is the canonical Wnt (wingless-type MMTV integration site family)/β-catenin signalling pathway. Upon binding of a Wnt ligand to its receptor (Frizzled), cytosolic β-catenin translocates to the nucleus where it forms complexes with transcription factors such as the TCF/LEF (transcription factor/lymphoid enhancer-binding factor) family and thereby regulates downstream target-gene expression. In absence of the Wnt signal cytosolic β-catenin is phosphorylated by GSK-3β (glycogen synthase kinase 3β) and subsequently degraded [130–132]. Members of the canonical Wnt/β-catenin signalling pathway are generally expressed during hypertrophy and accordingly also promote chondrocyte hypertrophy, presumably via the TCF/LEF binding site in the promoter region of the Runx2 gene [133–135]. In early chondrogenic differentiation Sox9 interacts with β-catenin and promotes its phosphorylation and thereby degradation thereby preventing osteoblastic or hypertrophic differentiation [133, 136–139].

In conclusion the process of endochondral ossification is dictated by spatiotemporal expression and function of variable transcription factors, ECM molecules and interacting regulatory molecules.

## 3.4 Importance of Cartilage Homeostasis in Outcome of Cartilage Repair

To maintain hyaline cartilage and prevent repaired cartilage from hypertrophic differentiation and as such further optimize cartilage repair approaches, local environmental factors need to be optimized. Such environmental factors are part of a healthy joint homeostasis which also enables hyaline cartilage to maintain its desired chondrogenic phenotype and prevent it from hypertrophic differentiation.

Joint homeostasis is described to be essential during cartilage repair, but methods for improving joint homeostasis in cartilage repair techniques are hardly addressed [140, 141]. An improved microenvironment may not only be the key to a new generation of bone marrow-based techniques to regenerate hyaline cartilage [142], but may also be a key factor for other progenitor cell based strategies and even cartilage repair in general. While inflammation is generally seen as a negative factor for joint homeostasis and are contributing factors in OA and rheumatoid arthritis (RA), it is also known to be the first and essential phase of tissue repair in general. Moreover, bone fracture healing depends on haematoma formation [143–145]. This suggests that inflammatory processes could be relevant pathways for addressing cartilage tissue repair. Supporting data for this notion is found in bone fracture healing processes where haematoma formation and injury-induced inflammatory responses are essential for fracture healing and its accompanying chondrogenic differentiation / endochondral ossification [143–145]. This essential inflammatory response induces local expression of extracellular signalling molecules like TGF-β1, BMPs, insulin-like growth factor (IGF)-1 and platelet derived growth factor (PDGF), which regulate chondrogenic differentiation processes [146, 147]. In addition, several inflammatory cytokines and chemokines (e.g. interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNFα), prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO)) are essential for bone fracture repair as well [144, 147–149].

### *3.4.1 Inflammatory Molecules and Chondrogenic Differentiation*

The general understanding on the role of inflammatory molecules in articular cartilage development, maintenance and osteoarthritic degradation is a katabolic one. Inflammatory processes that initiate and/or maintain the osteoarthritic status in an OA joint are thought to mainly originate from the synovium possibly reacting to cartilage breakdown products. Here synoviocytes produce inflammatory mediators that attack the cartilage matrix, causing infiltration of immune cells and finally affect cartilage viability and function. Important inflammatory molecules in the OA progression are e.g. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells), TNFα, interleukins and cyclooxygenases [150, 151]. Interestingly, despite the overall katabolic environment in an OA joint, osteoarthritis often induces osteophyte formation. Basically, these are ossifying and isolated ectopic cartilaginous tissues near the synovial membrane, which are committed to follow the process of endochondral ossification [152, 153]. The formation of cartilaginous osteophytes is in contradiction with the overall katabolic environment in the OA joint and it is therefore hypothesized in literature that precursor cells from the synovial or periosteal tissue are activated to undergo chondrogenic differentiation by mechanisms that are not fully understood yet [153], but do require TGFβ's for their induction [154, 155]. Recent reports show that the inflammation related NF-κB subunit p65 is an essential transcription factor for Sox9 and BMP-2 [156, 157]. Transcriptional

induction of BMP-2 by p65 was found to be essential for longitudinal bone growth via endochondral ossification [158]. Similarly, TNFα was found to induce expression of BMP-2 as well [159, 160]. Essentially, these previous reports for the first time explored the connection between inflammatory pathways and chondrogenic differentiation in an anabolic way, instead of the classic degenerative connection only. Further support for this new dogma was found by Aung and colleagues, who recently published that OA cartilage-conditioned medium is able to induce chondrogenesis of human bone marrow stem cells [161]. Chen *et al* confirmed this phenomenon *in vivo* by subcutaneous implantation of fibrin glue mixed with bone marrow stem cells (BMSCs) and osteoarthritic cartilage fragments [162]. It was found that, specifically in the presence of OA cartilage, BMSCs are induced to differentiate in the chondrogenic lineage. In addition, it was shown that human mesenchymal stem cells produce growth factors after stimulation with LPS or TNFα in a NF-kB dependent manner [163]. Together these reports suggest that OA chondrocytes excrete factors that induce chondrogenic differentiation of mesenchymal progenitor cells. Finally, recent work by the authors confirms the hypothesis that indeed external inflammatory factors (LPS, TNFα, etc.) are able to induce chondrogenic differentiation of progenitor cells, even without the addition of well-known chondrogenic growth factors (e.g. TGFβs or insulin) [164]. P65, COX-2 and iNOS are specifically expressed in the resting zone chondrocytes of the developing growth plate, indicating that an inflammatory process is involved in early chondrogenic differentiation. Furthermore, activated p65 was found to be a crucial factor in the induction of inflammatory molecule-driven chondrogenic differentiation, by initiating an early transient induction of Sox9. In addition to the classic Sox9 function in cartilaginous matrix synthesis, this novel Sox9 characteristic somehow relates to the very early initiation of chondrogenic differentiation via mechanisms that are still unknown. Taken together there is a recently growing body of experimental evidence, showing that inflammatory molecules and their down-stream pathways are not only associated with cartilage degeneration, but are also crucially involved in the initiation of the chondrogenic differentiation process. However, it is important to realize that this a very mild and temporarily action and takes place very early in differentiation and these same mediators could have very different, katabolic, actions later in chondrogenic differentiation/cartilage maintenance. These data suggest that for cartilage regenerative medicine one might make use of these inflammatory properties, as the initiation of chondrogenic differentiation is a crucial event for progenitor cell-based cartilage repair techniques.

### *3.4.2 Inflammatory Molecules and Cartilage Hypertrophic Differentiation*

Another interesting inflammatory phenomenon in the development of OA is being explored. For the articular cartilage component, osteoarthritis is in many ways similar to endochondral ossification, as in OA articular chondrocytes start to

differentiate into hypertrophic chondrocytes for reasons that are not yet completely understood [18–20]. Notably, the OA associated inflammatory factors and accompanying cell stress are known to be involved in chondrocyte hypertrophic differentiation in the growth plate and may explain why articular cartilage is terminally differentiating in OA [165]. Stress-related pathways that are activated in growth plate chondrocyte hypertrophic differentiation involve ER-stress/unfolded protein response, oxidative stress [166–168], advanced glycation end product formation (AGEs) [169–173], DNA damage and others [165]. In the growth plate these pathways are activated due to the rapid cell proliferation in the proliferative zone, reoxygenation of hypertrophic chondrocytes from the subchondral bone marrow, vast extra cellular matrix protein synthesis, etc [165]. Moreover, as a result of hypertrophic differentiation, these cells also start to express inflammatory molecules (COX-1, COX-2 [174], iNOS [175–177], p65 [158, 178, 179] and others (our unpublished data)), which are thought to enhance the intrinsic cellular capacity for hypertrophic differentiation. The message that should be taken from these observations is that failure of cartilage reparative and regenerative techniques due to formation of interlesional osteophytes, hypertrophic differentiation and calcification of cartilage grafts, may originate from similar processes. The pathways and phenomena stated above are therefore expected to be promising targets for avoiding failure due to terminal differentiation of the cartilage graft in the clinic.

We recently found that pharmacological inhibition of the key inflammatory enzyme cyclooxygenase-2 by e.g. Celecoxib decreases the level of chondrocyte hypertrophic differentiation, even in BMP-2 induced chondrocyte hypertrophy [174]. This may provide a potential pharmacological approach to prevent or decrease chondrocyte hypertrophic differentiation in cartilage repair techniques. Other authors have identified anti-oxidative components that decrease inflammatory signaling or chondrocyte hypertrophic differentiation. These components include N-acetyl cysteine [180–182], resveratrol [183–185], and even mechanical loading [165, 186]. Similarly, parathyroid hormone related peptide (PTHrP) is known for its capacity to keep proliferating articular chondrocytes in their chondrocyte state and prevent them for further developing into hypertrophic chondrocytes [15, 187]. In conclusion, targeting inflammatory mediators and stress related pathways may thus provide a potential pharmacological approach to prevent or decrease chondrocyte hypertrophic differentiation in cartilage repair techniques.

## 3.5 Conclusion

In summary, it now becomes clear that inflammatory signaling is not only involved in cartilage degradation, but is also indispensable for initiating the differentiation of chondrocytes from progenitor cells on, albeit it in a very mild and temporarily action. Thereby this brings a whole new view on the role of inflammatory mediators and their link to cartilage in general. Especially for progenitor cell based repair technologies, these new insights could be employed to increase the differentiation

potential of progenitor cells toward engineered cartilaginous tissue *in vitro and in vivo*. Furthermore, part of the failure of cartilage repair techniques originates from calcification or hypertrophic differentiation of the cartilage graft, as well due to the development of interlesional osteophytes. The authors believe that part of these adverse effects might be avoided when joint homeostasis which is ideal for the different phases of regeneration or repair is also taken into account as an important factor in the post-operative treatment strategy after cartilage repair. A synovial fluid environment supplemented with the aforementioned factors might contribute to the success rate on an anti-hypertrophic basis possibly for both the chondrogenically differentiating cells as well as the subchondral bone. Additionally it could also be envisioned that any joint homeostasis-disturbing intervention could benefit from an approach where joint homeostasis which is optimal for cartilage repair is recognized as a prerequisite for success. For these, anti-oxidative and anti-hypertrophic agents could play an important role to achieve this goal as well. In addition, since hypertrophy and ossification are also believed to be essential underlying processes in the process of OA these findings may also be of concern in the process of OA.

**Acknowledgements** This work is financially supported by the Dutch Arthritis Association (grant LLP14) (http://www.reumafonds.nl) and the Dutch Stichting Annafonds|NOREF (grants 07/07 and 08/42) (http://www.annafonds.nl). The authors also thank D.A.M Surtel and A. Cremers.

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