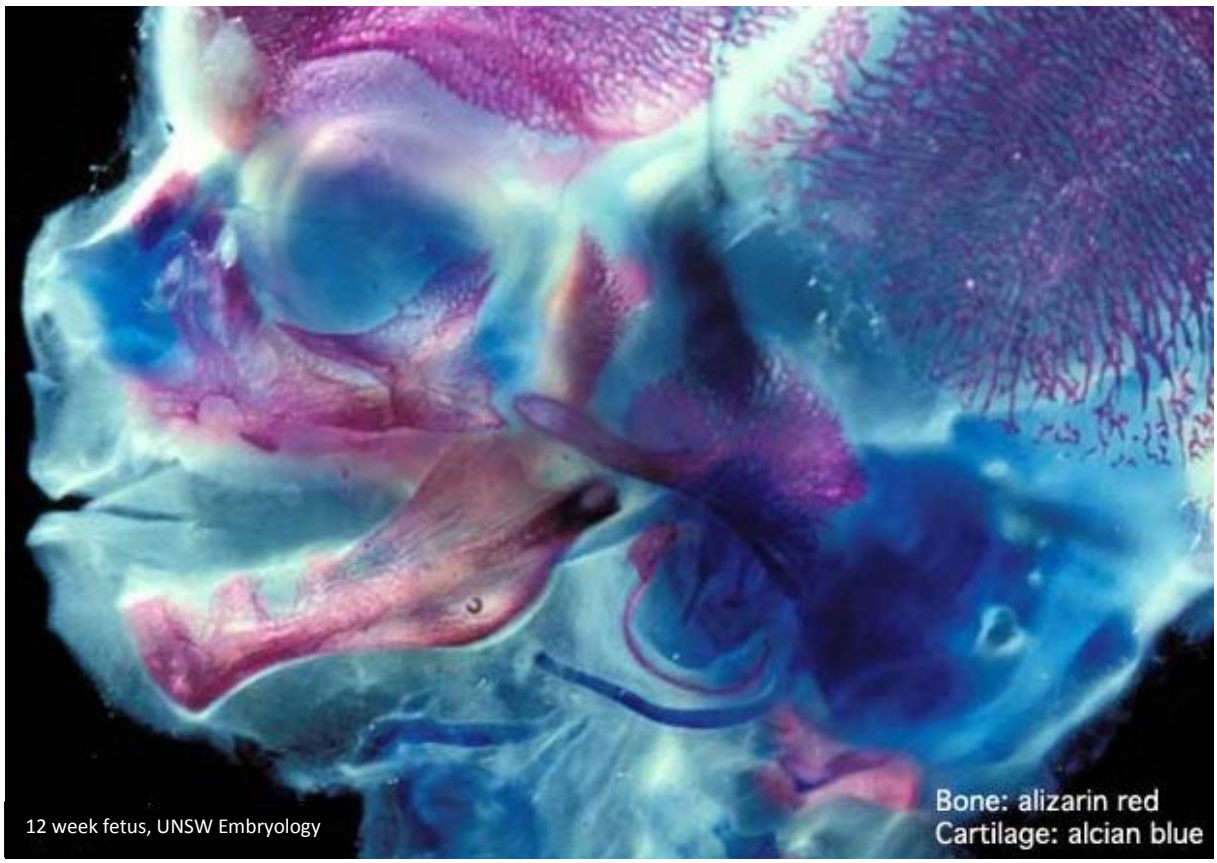
# Can vascularization and embryonic development be used as cartilage defect repair mechanisms?

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*Introduction*

Cartilage is a tough, flexible and elastic connective tissue with a primary function to disperse forces on the joints during movement. Cartilage is an avascular structure which is mainly composed of a collagen and proteoglycan-rich extracellular matrix (ECM) with its primary cell type, the chondrocyte[1]. Defects cause pain and result in decreased mobility and can occur either through sudden mechanical damage or over a longer period of time by slow degeneration. The degenerative joint disease osteoarthritis (OA) is age related and mainly affects the joints that are under continues stress throughout the years like knees, hips and fingers. It is estimated that 9.6% of men and 18.0% of women worldwide over the age of 60 have OA symptoms. With 80% of OA patients having limited mobility and 25% that cannot perform their daily activities, OA is in the top ten of disabling conditions in developed countries (WHO, chronic rheumatic conditions, viewed August 2012). Endogenous cartilage repair is limited and small defects can therefore degenerate over time leading to more severe conditions like OA[2].

Current repair strategies are able to improve joint function but are not able to fully repair a defect over a longer period of time[2]. Either the current treatments have to be improved or other repair mechanisms should be developed to fully repair defects for a longer period of time.

In the current paper cartilage development, the factors involved in cartilage formation and current cartilage defect treatments will be described. However, the main focus will be whether embryonic development can be used as a cartilage repair mechanism. Are we able to reactivate embryonic cartilage development without subsequent mineralization and bone formation? Which factors in cartilage development could be used for this reactivation? Will vascularization of the cartilage aid repair and how can we achieve this vascularization?

*Cartilage formation and endochondral ossification*

Bones in our body are formed by two mechanisms: intramembranous and endochondral ossification. Whereas intramembranous ossification forms flat bones, like the bones of the skull, endochondral ossification generates the long bones in our body. During endochondral ossification a cartilage model is formed prior to bone formation. This cartilage model is formed through condensation of mesenchymal cells, which originate from the lateral plate mesoderm, neural crest or the somites, and migrate towards the limb field[1]. This condensation of undifferentiated mesenchymal cells is called pre-cartilaginous condensation. The pre-cartilaginous condensation produces an ECM rich in collagen type I, hyaluranon, tenascin and fibronectin[3]. Their differentiation into chondrocytes, chondrocyte proliferation and hypertrophy, the excretion of cartilage extracellular matrix and its mineralization finishes the cartilage model and opens the road for subsequent bone formation[4, 5]. Upon differentiation into chondrocytes, a different composition of ECM is produced, rich in cartilage specific collagen type II, collagens type IX and XI, Gla protein, the large chondroitin sulfate rich proteoglycan, aggrecan, and link protein. The expression of collagen type I is turned off. The hypertrophic chondrocytes produce high levels of alkaline phosphatase and collagen type X and diminished levels of collagen type II and IX compared to proliferative chondrocytes[3, 6]. Two sites of growth can be described on either end of the developing bone: the growth plate, separates the primary and secondary ossification centers, and the articular-epiphyseal growth cartilage (AEGC). The chondrocytes in both growth areas are arranged in morphological distinct zones. Furthest from the ossification front, the zone where cartilage is replaced by bone, is the zone of resting chondrocytes. Next to the resting chondrocytes lies the zone of proliferation. Here, round proliferative chondrocytes are flattened and stacked to form columns. After proliferation the chondrocytes go

through a pre-hypertrophic and hypertrophic state were they hypertrophy and produce a different composition of ECM then was produced before[3, 6]. The proliferation and ECM excretion is the actual process of bone elongation. Subsequent to hypertrophy the chondrocytes die and as they do the transverse septa of ECM between them is broken down. The vertical septa between the columns of chondrocytes remain and are later removed. The death of the chondrocytes and removal of the transverse septa allows infiltration of cells of the ossification front: blood vessels, osteoclasts, precursors of osteoblasts and bone marrow cells. The osteoclasts will assist in the removal of the remaining cartilage whereas the osteoblasts use the remaining cartilage model for the deposition of bone matrix. At puberty the ossification front overruns the growth plate and the bone of the primary ossification front (metaphysis) and the bone of the secondary ossification front (epiphysis) fuse, forming mature bone[5]. The cartilage that will remain is the articular cartilage on both distal ends of the bone. This cartilage will function as a shock absorber and lubricate the joints. Articular cartilage consists of four different layers: the superficial layer, the transitional layer, the deep radial layer and the calcified cartilage layer (CCL). The CCL is located at the interface between the deep cartilage and the subchondral bone. It anchors the joint cartilage to the underlying bone surface. The boundary between the deep cartilage and the CCL is visible as a basophilic line called the tidemark, which is the mineralizing front. Chondrocytes along the tidemark are hypertrophic, produce alkaline phosphatase, which allow mineralization of the ECM and type X collagen[7]. Between the trabecular bone and the CCL is the subchondral plate. The subchondral plate is a highly vascularized plate with the main function to absorb mechanical loads applied to the joint and provide the cartilage with about 50% of the required nutrients through the terminal blood vessels which are in direct contact with the deep cartilage layer[7].

*Embryonic limb development*

Development of the vertebrate limb is a complex interaction of proteins, reviewed by DeLise *et al.,* which starts with the recruitment of mesenchymal cells to the limb field. The mesenchymal cells will start to condense to form the pre-cartilaginous mesenchyme. The still undifferentiated mesenchymal cells produce an ECM rich in collagen I, hyaluranon, tenascin and fibronectin[3], as mentioned before. This composition of ECM inhibits close cell-cell interactions. Since pre-cartilaginous condensation is crucial for the onset of chondrogenesis and requires intimate cell-cell and cell-matrix interactions, the production of such a matrix delays chondrogenesis. When condensation begins the mesenchymal cells start to produce hyaluronidase which decreases hyaluranon concentration and allows for stronger cell-cell interactions. Two cell adhesion molecules have been described in their importance in cell condensation: neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM). Besides these cell-cell interactions the cell-matrix interaction also seems to be important in cell condensation. An ECM component which is implicated in this interaction is fibronectin, since expression is increased in areas of cellular condensation[3, 6, 8].

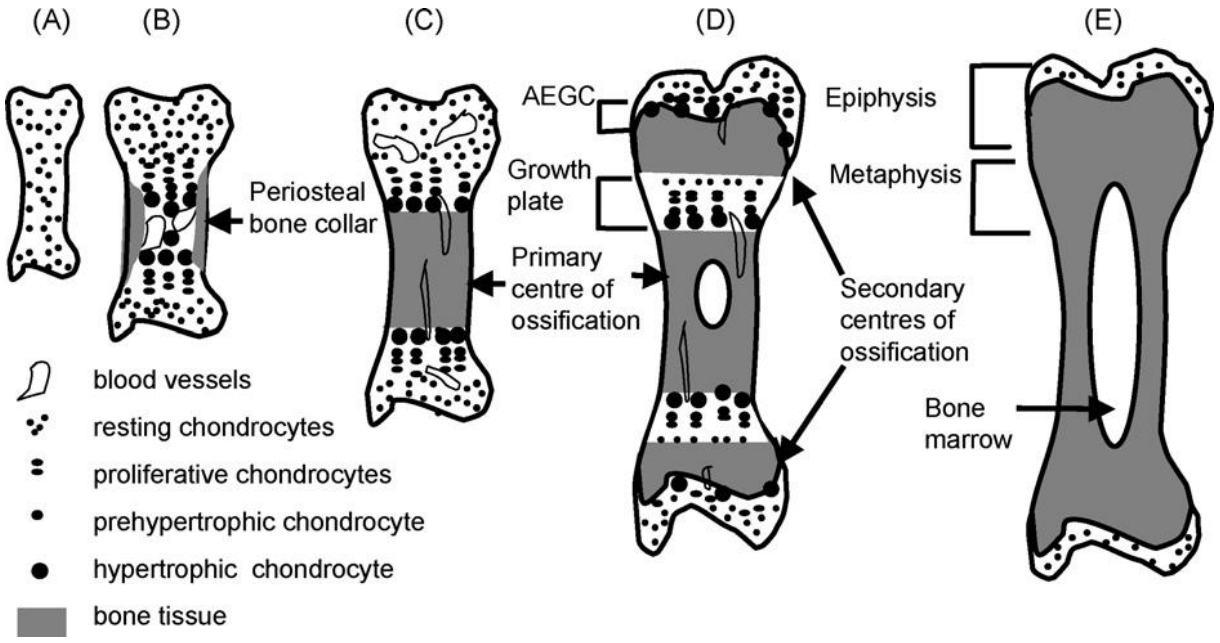


Fig. 1: Schematic overview with the different stage of endochondral ossification. A, Cartilage model. B, Chondrocyte proliferation and hypertrophy, formation of the primary centre of ossification and the invasion of vasculature. C, Growing primary centre of ossification. D, Formation of secondary centres of ossification separated from the primary centre by the growth plates. The AEGC remains under the permanent articular cartilage. E, in the adult bone the growth plate is lost due to fusion of primary and secondary ossification centres. The AEGC is replaced by bone and only the articular cartilage remains. Mackie *et al.* (2008)

Multiple secreted factors are involved in limb development and include growth factors such as fibroblast growth factors (FGFs) and transforming growth factors (TGFs), the Wnt family of secreted glycoproteins and transcription factors such as engrailed (En), sonic hedgehog (Shh), radical fringe (r-Frg) and *msx-1 and msx-2* which are encoded by the homeobox (Hox) gene family[3].

Limb formation is initiated by the expression of *fgf-10* and its possible interaction with *fgf-8*. The ectoderm thickens and forms a specialized epithelium called the apical ectodermal ridge (AER). The AER is induced and maintained by members of the FGF family. Cells directly underneath the AER rapidly proliferate and maintain characteristics of undifferentiated mesenchyme, this zone is termed the progress zone (PZ)[3].

The formation of the three dimensional structure of a limb can be described by development along three axes: the proximodistal, dorsoventral and anterioposterior axis. Each axis has a different set of proteins involved. The proximodistal axis is defined by FGFs. FGF-2, -4 and -8 are indicated in outgrowth of the limb. Furthermore, members of the bone morphogenetic protein (BMP) family, *bmp-2, -4 and -7* may maintain the AER and PZ or induce FGF. The dorsoventral axis by the interaction of Wnt-7A and the En-1 transcription factor. The anterioposterior axis is largely dependent on Shh and the Hox genes. Shh is activated by FGF-4 from the AER and in turn regulates *bmp-2, -4 and -7* and possibly the expression of *gli-1* and *-3*[3].

*Factors influencing cartilage formation and endochondral ossification*

Multiple factors are known to influence cartilage formation and endochondral ossification, both locally produced and systemic as well as transcription factors and factors of the ECM. Locally produced factors including BMPs, FGFs, TGFβ, Wnts, Indian hedgehog (Ihh), parathyroid hormone-

related peptide (PTHrP) and retinoids. Systemic factors include growth hormone (GH) and thyroid hormone and transcription factors such as Gli3, sex determining region Y box gene 9 (Sox9), Runx2, myocyte enhancer factor-2 (MEF2C) and histone deacytelase-4 (HDAC4). An overview of factors involved in endochondral ossification and their interplay is shown in figure 2[3, 5].

The signaling molecules Ihh and PTHrP have been identified as regulators of chondrocyte maturation and hypertrophy[3].

Ihh is a secreted factor expressed by prehypertrophic chondrocytes, it stimulates proliferation and inhibits hypertrophy. When Ihh binds to its surface receptor, patched-1 (Ptc), phosphorylation and proteolytic processing of the Gli family of transcription factors is inhibited, thereby inhibiting their activity as repressors, thus proliferation is stimulated. Inhibition of hypertrophy is achieved by induction of expression of PTHrP. Since in the absence of both Ihh and Gli3 chondrocyte proliferation, PTHrP expression and rate of hypertrophy are normal, another pathway must regulate this independently of Ihh and Gli3. It is likely that IGFR1 signaling takes this role. Ihh and IGFs have similar effects on skeletal growth and experiments showed an additive but independent effect of Ihh and IGFs on growth cartilage[5, 9].

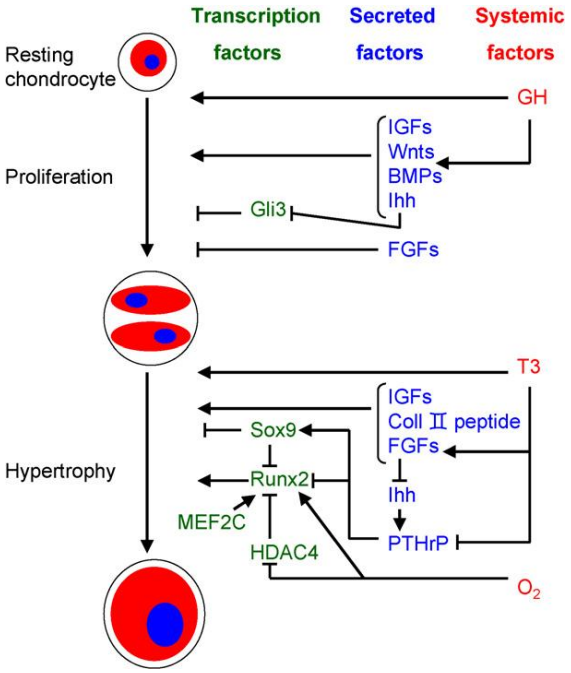


Fig. 2: Schematic overview of roles of systemic factors, locally secreted factors and transcription factors in chondrocytes proliferation and hypertrophy during endochondral ossification. Arrows indicate a stimulatory effect were as crossed lines indicate an inhibitory effect. Coll II: collagen type II.
Mackie *et al.* (2008)

PTHrP is structurally homologous to parathyroid hormone (PTH) and is expressed by perichondral cells and early proliferating chondrocytes. Both PTH and PTHrP act on PTH/PTHrP receptor-bearing cells. This receptor is expressed at low levels by proliferating chondrocytes and at significantly higher levels when these cells stop proliferating. PTHrP retains cells in a proliferative state and prevents hypertrophy. Limiting the transition from proliferation to hypertrophy, PTHrP limits the amount of cells expressing Ihh and thus regulates its own expression and the rate of chondrocyte differentiation. Since PTHrP is required to diffuse to exert its effects, the proliferative zone will only extent as far as where the concentration PTHrP is sufficient to prevent the transition from proliferation to hypertrophy. The ability of cells furthest away from PTHrP expression to escape from PTHrP control will initiate Ihh expression which in turn maintains PTHrP expression and this inhibits the transition to hypertrophy[5].

PTH/PTHrP receptor activation leads to the production of cAMP and subsequent protein kinase A (PKA) activation. PKA will phosphorylate and activate Sox9. Sox9 is suspected to delay chondrocyte hypertrophy. PTH inhibits the expression of Runx2, a stimulator of chondrocyte hypertrophy, after activation of the PTH/PTHrP receptor[5].

Since PTHrP expression is elevated in hypothyroid rats and PTH/PTHrP receptor expression is suppressed in rats with excessive levels of circulating thyroid hormone it could be hypothesized that thyroid hormone exerts its effect on chondrocytes, at least in part, by suppressing PTH/PTHrP receptor signaling[5].

*Cartilage vascularization*

Vascularization of cartilage is a key process in ossification of the articular cartilage[10, 11] and is related to osteoclast invasion, formation of the primary center of ossification and thus bone formation[5, 6]. The invasion of vessels during development and regulation of the avascular structure of cartilage during life is a balance between pro- and anti-angiogenic factors shown in table 1[6] and matrix remodeling[12]. Apart from the factors listed in table 1 transcription factors also play an important role in cartilage vascularization[13]. The pro- and anti-angiogenic and transcription factors will be discussed below.

The first and most commonly known factor is VEGF. VEGF is a well-known angiogenic stimulator in a

**Table 1. Angiogenic and anti-angiogenic factors expressed by chondrocytes in the growth plate**

| *Growth factor* | *Angiogenic effect* | *Site of expression* | *Target cell type/receptor expression* |
|---|---|---|---|
| CTGF | Pro | Hypertrophic chondrocytes<br>Endothelial cells | |
| IGF-1 | Pro | Proliferative chondrocytes | Proliferative chondrocytes |
| EGF | Pro | Proliferating chondrocytes | |
| PDGF-A | Pro | Mature, non-hypertrophic chondrocytes | Hypertrophic chondrocytes, sites of vascular invasion |
| Transferrin | Pro | Hypertrophic chondrocytes | Endothelial cells |
| Cyr61 | Pro | Differentiating chondrocytes | NA |
| VEGF | Pro | Hypertrophic chondrocytes<br>Embryonic lower hypertrophic chondrocytes | Endothelial cells<br>Chondroclasts<br>Osteoblast |
| Unidentified | Pro | Hypertrophic chondrocytes | Endothelial cells |
| MMP-9/gelatinaseB | Pro | Chondroclast | Endothelial cells |
| Acidic and basic FGF | Pro | Hypertrophic chondrocytes | Hypertrophic chondrocytes<br>Endothelial cells |
| TGF-β1 | Pro/anti | Chondrocytes | Microvascular endothelial cells |
| TSP-1 | Anti | Early chondrocytes | |
| TSP-3 | Anti | Proliferative and hypertrophic chondrocytes | |
| TIMP-1, 2 | Anti | Chondrocytes | Endothelial cells |
| ChM-I | Anti | Chondrocytes | Endothelial cells |
| CHIAMP | Anti | Chondrocytes | Endothelial cells |

Gerber *et al.* Cardiovasc Med (2000)

variety of *in vivo* models by promoting growth and migration of vascular endothelial cells and protects them from apoptosis[14, 15]. Three isoforms of VEGF are known: $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ in mice ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in humans). $VEGF_{120}$ is soluble, $VEGF_{188}$ binds to the cell surface and ECM and $VEGF_{164}$ combines these two characteristics[16]. VEGF is expressed by hypertrophic chondrocytes in the epiphyseal growth plate[14, 17] and by osteoblasts and osteoclasts[7] but not by resting chondrocytes[6]. Two tyrosine kinase receptors are known to be affected by VEGF, VEGFR-1 (flt-1) and VEGFR-2 (flk-1). While VEGFR-1 is implicated in chemotaxis and vascular permeability, VEGFR-2 is implicated in endothelial cell proliferation and survival[15]. *Vegf* expression can be regulated by IGF-I, PTH, TGF-β and FGF-b[10]. However, a major VEGF regulator is the hypoxia-inducible factor-1α (HIF-1α)[18]. It has been shown that VEGF inactivation results in complete disruption of the vascular

network of the growth plate in juvenile mice[19]. Furthermore, it is suggested that the soluble VEGF isoforms are required for normal vessel attraction[16]. VEGF may also facilitate the secretion of matrix metalloproteinases (MMPs) and reduce the production of tissue inhibitor of metalloproteinase (TIMPs)[20].

A major player in keeping cartilage avascular is chondromodulin (ChM)-1. ChM-1 is a glycoprotein that inhibits endothelial cell proliferation. Kusafuka *et al.* postulated that ChM-1 is located in the interterritorial matrix of cartilage and since ChM-1 is a strong angio-inhibitor it acts as a barrier against the angiogenic properties of VEGF and FGF-2[21]. The loss of ChM-1 coincides with the invasion by blood vessels, the upregulation of expression of VEGF, MMP-9 and MMP-13[7].

Apart from pro- and anti-angiogenic factors matrix remodellers play an important role in vessel invasion by degrading the cartilage[22].
Before vascular invasion can occur the vascular basement membrane has to be degraded and the ECM remodelled in order for endothelial cells to migrate. The dominant MMPs involved in this process are MMP-2, MMP-7 and MT1-MMP. These enzymes release the angiogenic factors VEGF and FGF-2 from the ECM and expose binding site for pro-angiogenic integrins[7]. Furthermore, the breakdown of the basement membrane primes the endothelial cells for proliferation and migration[23]. Experiments have shown that incubation of hypertrophic cartilage in a collagen co-culture system with endothelial cells, an angiogenic response was observed in wild type cartilage but not in GelB(-/-) cartilage[6].
MMP-13 is required for collagen type 2 degradation and together with MMP-9 the degradation of aggrecan. Aggrecan degradation is required in order for collagen type 2 to be degraded[22].
MMP-9/gelatinase B is expressed by chondroclasts and degrades components of the ECM such as collagens type XI but not collagen type I, proteoglycans (it can in synergy with MMP-13) or laminins which is required for vascular invasion and bone formation[24]. However, apart from the pro-angiogenic effects of MMPs anti-angiogenic effects of MMPs are also seen due to release of anti-angiogenic factors released when the ECM is degraded [7].

Connective tissue growth factor (CTGF) is important player in growth plate chondrogenesis, angiogenesis and bone formation/mineralization. CTGF is produced by hypertrophic cartilage and invading endothelial cells[25]. It has also been shown to be implicated in cellular proliferation, migration, adhesion, survival, differentiation, ECM protein synthesis and more complex processes such as angiogenesis, chondrogenesis, osteogenesis[26]. CTGF can bind and is directly stimulated by TGF-β1 as well as other factors, such as BMP-2, and is secreted into the ECM. It has been also shown that CTGF can inhibit BMP receptor signalling by its interaction with BMP-4[26]. The role of CTGF in skeletogenesis is emphasized by CTGF-null mice which show impaired growth plate chondrogenesis, angiogenesis and bone formation/mineralization. Recent studies have showed that CTGF is required for pre-cartilage formation by stimulating mesenchymal cell proliferation, migration and condensation [26]. Furthermore, absence of CTGF results in inhibition of terminal differentiation of chondrocytes[26]. In contrast to these actions, CTGF is thought to form a complex with $VEGF_{165}$, thereby blocking the pro-angiogenic activity of VEGF and MMPs are able to degrade CTGF in this complex activating VEGF[7].

IGF-I has been shown to be the main anabolic growth factor for articular cartilage and plays a role in cartilage homeostasis, balancing proteoglycan synthesis and breakdown by the chondrocytes. Both IGF-I levels and chondrocyte responsiveness to IGF-I diminish with age, suggesting that the ability of cartilage to maintain its structural and functional integrity decreases with age. This diminished responsiveness to IGF-I has also been observed in arthritic and inflamed cartilage. The decrease in responsiveness appears to be due to, at least in part, overexpression of IGF binding proteins[27].

Epidermal growth factor (EGF) is part of the EGF family and is localized in the resting and proliferative zone[28] and the EGF receptor (EGFR) is localized on vascular endothelial cells[29]. Inhibition of the EGFR was shown to inhibit endothelial cell proliferation and vessel formation in mice with rheumatoid arthritis[29]. Another member of the EGF family, TGF-α, also has the ability to bind and activate the EGFR. [30]. Both EGF and TGF-α negatively regulate chondrogenesis of mesenchymal cells during embryonic development[30].

Platelet derived growth factor (PDGFs) is a locally produced and acting growth factor which is, among others, synthesized by endothelial cells and primarily stored in platelets. It consists of two distinct disulphide linked peptide chains, A and B, and can be either a homo- or heterodimer. PDGF plays a fundamental role in wound healing cascade and is a potent mitogenic and chemotactic factor for cells of mesenchymal origin, including chondrocytes. Furthermore, PDGFs induce chondrocyte proliferation and differentiation[31]. Indirect evidence of the involvement of PDGF in cartilage can be seen in cartilage defects treated with microfracture. Upon clot forming in the defect PDGF is released in the defect site exerting mitogenic and chemotactic effects on the surrounding cells in the cartilage. The PDGF receptor has been found on a number of cell types including chondrocytes[27].
Treatment with PDGF-BB, inhibits resting chondrocyte maturation and incubation with PDGF-BB in culture appeared to increase chondrocyte proliferation and cartilage proteoglycan production while inhibiting progression to maturation[27].

FGF-2 is suggested to inhibit hypertrophy at high concentrations and to be an angiogenic factor. The FGF receptors FGFR1 and FGFR3 are both implicated to promote vascular invasion and ossification of the hypertrophic zone and are thus negative regulators of longitudinal bone growth. Whereas FGFR2 has been implicated to be a positive regulator of endochondral ossification[32]. Both FGF-18 and FGF-9 are indicated to interact with FGFR1 and to induce the expression of VEGF and VEGFR1[33].

TGF-β is part of the TGF-β superfamily, which, among others, also includes BMPs[34]. TGF-β has been related to angiogenesis and bone remodelling and is reported to be secreted by chondrocytes during endochondral ossification[21] and cartilage is even described as one of the major sources of TGF-β[35].

Thrombospondin (TSP)-1 is a glycoprotein involved in chondrocyte-ECM interaction and is found in the transitional and deep cartilage layers. TSP-1 activates TGF-β1 and inhibits angiogenesis by preventing endothelial cell migration, inducing endothelial cell apoptosis and inhibiting VEGF to bind to the VEGFR2 receptor[7]. TSP-2 mRNA is concentrated in condensating mesenchymal cells, whereas early chondrocytes express TSP-1 and more mature chondrocytes express TSP-3. Furthermore, TSP-1 has been shown to be able to bind and activate TGF-β1[36].

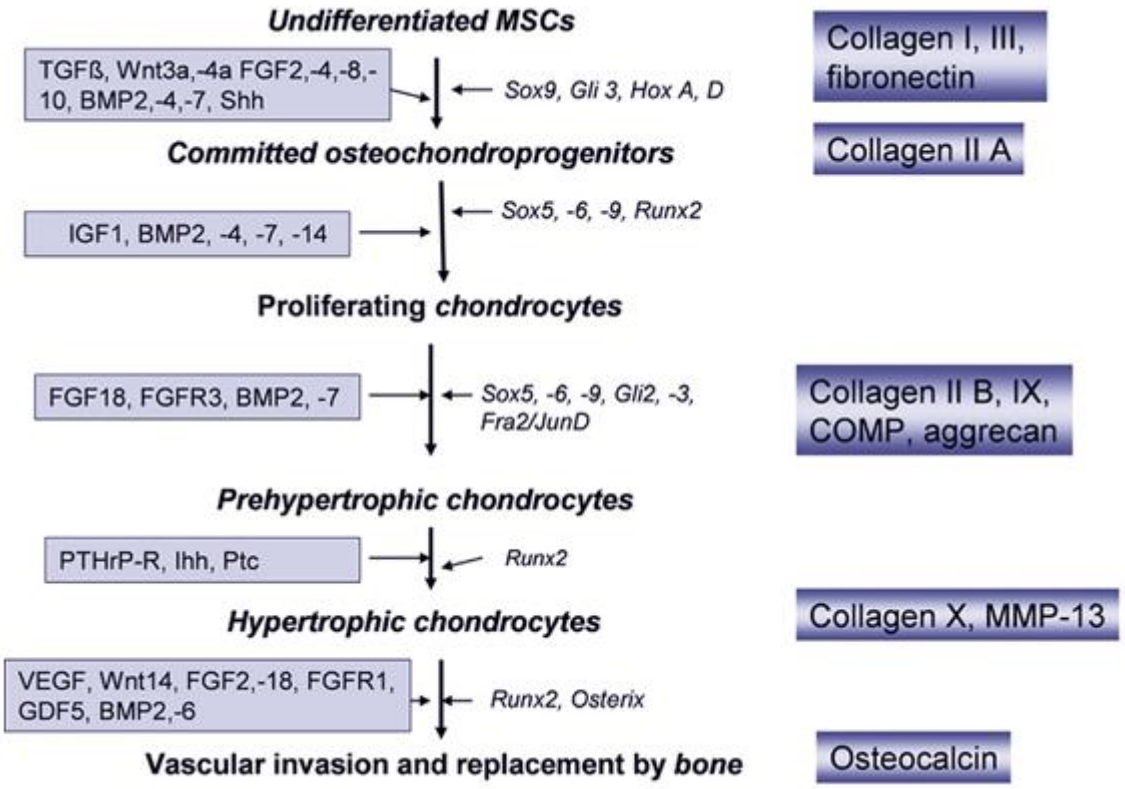


Figure 3: Chondrogenic differentiation cascade with growth factors, signaling molecules and differentiation markers. Grassel S. and Ahmed N. (2007)

Other anti-angiogenic factors include the chondrocyte inhibitor of angiogenesis (hCHIAMP), TIMP-1 and TIMP-2. The mechanisms that regulate the expression of these anti-angiogenic factors within cartilage are still unknown[7]. Their mechanisms are difficult to investigate since conventional knock-out experiment showed early embryonic lethality before chondrocyte hypertrophy[6].

*Transcription factors*

As stated before transcription factors also play a role in cartilage vascularization.

Sox9 is expressed in resting, proliferative and pre-hypertrophic chondrocytes but not in hypertrophic chondrocytes and regulates the expression of cartilage specific genes such as *Col2a1*, *Col9a1*, *Col11a1* and aggrecan[5, 13]. Hattori *et al.* demonstrated that overexpression of *Sox9* in hypertrophic chondrocytes is a major negative regulator of cartilage vascularization and bone formation. This regulatory effect is, in part, due to inhibition of the expression of V*egfa* and *Mmp13,* which are normally upregulated in hypertrophic chondrocytes[13]. Furthermore, overexpression of *Sox9* resulted in increased collagen type 2 and proteoglycan expression[37]. SOX9 is also know to activate aggrecan gene 1 (Agc1) and other cartilage-specific genes[38].

Runt-related transcription factor 2 (Runx2) is required for vascularization and ossification of the cartilage. In the absence of Runx2 in Runx2$^{-/-}$ mice there is no maturation of chondrocytes and vascularization[39]. Runx2 is assisted in his actions by Runx3[5] and KO of both Runx2 and Runx3 completely lack *Ihh* expression[39].

*Current cartilage defect treatments*

Cartilage damage can occur mechanically due to an accident or by a degenerative disease such as osteoarthritis (OA). The degenerative disease OA is associated with an imbalance between anti- and pro-angiogenic factors and blood vessel invasion. Some characteristics of OA even resemble normal endochondral ossification during skeletal development. During OA there is articular chondrocyte proliferation, expression of hypertrophy markers (MMP-13 and collagen X), remodelling of the cartilage matrix, vascularization and focal calcification of joint cartilage[9]. The defect can be of partial thickness, indicating the defect has not penetrated to the subchondral bone, or a full thickness defect in which it has penetrated the subchondral bone[2]. Unfortunately, due to the avascular and aneural structure, with restricted blood, oxygen and nutrient supply, endogenous regeneration is a rare event[40, 41]. Due to restricted blood flow, progenitor cells cannot reach the injured areas. Next to this, the surrounding chondrocytes do not migrate into the cartilage defect and produce a reparative matrix. Since cartilage contains only a small volume of cells which could be able to aid in the repair, this also forms a major problem. However, there is some attempt at repair when the damage breaches the underlying subchondral bone. A primitive mesenchymal blood clot forms which leads to the production of fibrocartilage. This fibrocartilage has different, inferior mechanical characteristics compared to articular hyaline cartilage and is therefore not the best solution[40]. Due to this limited repair in articular cartilage a small defect may, over time, develop into a large, severe defect which requires extensive treatment and even surgery.

A defect in articular cartilage causes two problems, the void in the damaged cartilage needs to be filled with a tissue with the same mechanical properties as the surrounding articular cartilage and the replaced tissue needs to integrate with the remaining cartilage[2]. Multiple strategies exist in repairing damaged cartilage: arthroscopic repair procedures like debridement, soft tissue grafts, osteochondral transfer, cell transplantation-based repair and the use of stimulatory factors such as growth factors, cytokines or transcription factors. However, most treatments only alleviate pain and prosthesis of the joint is often the final step in a treatment. Therefore, cartilage repair is in need of improvement[2].

Arthroscopic debridement is a technique used to “clean” an injured area. The damaged cartilage is removed so that only healthy cartilage remains. However, this does not promote repair and will only relief pain. Therefore, debridement is often used prior to other techniques which are aimed at repair, like microfracture. Microfracture is a technique by which small holes are made in the subchondral bone to induce bleeding. The bleeding will cause the formation of a blood clot and the formation of fibrocartilage, as mentioned before. Although the inferior fibrocartilage is formed which makes microfracture treatment not a curative one, it does provide pain relief for many years[2, 42].

Another treatment is osteochondral transfer, by which either autogenic or allogenic tissue is transferred to the damaged area. In autologous transfer a cylindrical plug is removed from non-load bearing regions of the articular cartilage and transplanted into the debrided defect. Results have shown decreased pain and improved joint function. However, it does require removing cartilage from another part of the body. Although the grafts are taken from a non-load bearing site, investigation showed that the non-load bearing regions are not truly non-load bearing. Therefore, harvesting grafts from these sites may influence their function over time as well[2].

Cell transplantation might be more useful in repairing the cartilage since specific cells can be chosen to repair the defect. An example is autologous chondrocyte implantation (ACI), chondrocytes are isolated from healthy cartilage and expanded in culture. First, a defect is debrided and the cultured

chondrocytes are injected in the defect. Although relieve of pain and restoration of function was seen, the beneficial effect is most likely not the effect of the transplanted chondrocytes since untreated defects show similar results[2]. Since chondrocytes are from mesenchymal origin the use of mesenchymal stem cells (MSCs) has been widely studied. Either injection of MSCs alone or with the use biodegradable scaffolds.

During the vertebrate life three different mechanisms of cartilage vascularization can be observed. The first is called quiescent angiogenesis. During late embryonic development, vascular structures invaginate from connective tissue surrounding the bone (perichondrium) into the bone through pre-existing cartilage channels. The second is the invasion of capillary structure at the growth plate regions during rapid postnatal growth. Third is the transient turn on of angiogenesis during injury or other pathological conditions after the growth plate has closed[6] as before growth plate closier vascularization is required for bone development.

*New strategies in cartilage repair*

Autonomous repair is minimal and usually results in inferior fibrocartilage. Although cartilage repair strategies are improving they still do not result in long term repair. Small defects remain and will degenerate into more severe defects over time resulting in the requirement of a joint prosthesis. However, a small synthetic molecule called kartogenin, has shown to be able to stimulate endogenous stem cells to differentiate and proliferate as matrix producing chondrocytes, with Runx1 as a downstream target[43]. Kartogenin displaces core-binding factor α (CBF-α) from filamin A which in turn is able to migrate to the nucleus and bind Runx1 and activate the production of proteins involved in differentiation and increase the synthesis of cartilage matrix protein such as collagen type 2, aggrecan and TIMPs[43].

A possible way to repair cartilage is to vascularize the cartilage in order for MSCs to migrate to the defect and differentiate into chondrocytes. This could be achieved by either vascularizing the existing cartilage or reactivating embryonic development patterns.

During cartilage development the cartilage is vascularized by invasion of blood vessels from the perichondrium after hypertrophic chondrocytes begin expressing unwanted collagen type X and decrease the production of collagen type II[3].

VEGF is a major factor for normal vascular ingrowth during endochondral ossification[44]. However, ChM-1 is present at high levels in the cartilage and acts as a barrier against the angiogenic actions of VEGF[21]. Therefore, in order to vascularize the cartilage ChM-1 should be downregulated or its actions inhibited. This will convert the scale in favor of VEGF and thus vessel formation. However, the actions of ChM-1 are associated with inhibition of ossification[45, 46] and inhibition of ChM-1 could therefore lead to unwanted ossification. Besides VEGF and ChM-1. MMPs also play an important role in vascularization in the sense that the matrix needs to be broken down in order for cartilage channels to be formed and vessel invasion to occur[20]. MMP-9 and -13 are crucial for vessel invasion[22] and therefore regulation of these MMPs is required in order to use vascularization for repair. Upregulation of both MMPs favors aggrecan and collagen type 2 breakdown and vessel invasion. However, aggrecan and collagen type 2 are the ECM molecules which need to be produced and breakdown of the molecules in order to vascularize the cartilage to aid in repair might be counterproductive.

Since CTGF is thought to be able to form a complex with VEGF[7], inhibition of CTGF could also result in more vascularization of the cartilage. Furthermore, absence of CTGF has been shown to inhibit terminal differentiation of chondrocytes[26] which would inhibit unwanted ossification of the cartilage.

The reactivation of embryonic development patterns could lead to condensation of MSCs and subsequent chondrogenesis. The MSCs have to be directed to the defect site and oxygen and nutrients need to be available in sufficient amounts. This could be achieved by vascularizing the cartilage. However, it must remembered that firstly, normal endochondral ossification and OA are associated with an imbalance of anti- and pro-angiogenic factors and vascularization of the cartilage[9]. Secondly, terminal differentiation of MSCs/chondrocytes has to be avoided since it will lead to ossification. It has been shown that viral gene transfer of BMP-2 and -4 efficiently induce MSC differentiation and delayed introduction of PTHrP or FGF-2 *in vitro* reduced the expression of collagen type 10a but unfortunately also that of collagen type 2 mRNA[47]. Cha *et al.* showed that dedifferentiated chondrocytes, a common reaction seen in chondrocyte cultured in 2D, with BMP-2 and Sox-9 gene transfer, redifferentiated the dedifferentiated chondrocytes *in vivo*[41].
As stated before, inhibition of CTGF could aid in the vascularization and inhibit terminal differentiation of chondrocytes. However, CTGF is also required for the pre-cartilaginous condensation of mesenchymal cells[26] and inhibition of CTGF could therefore work against MSC targeted repair. During life the cartilage deteriorates due to diminished responsiveness to IGF-I by increased IGF-I binding proteins[27]. Since IGF-I is related to maintaining the structural and mechanical properties of cartilage[27], increasing either the responsiveness to IGF-I or IGF-I levels this could aid in repair. Furthermore, PDGF-BB was shown to increase chondrocyte proliferation while inhibiting maturation.

*Conclusion*
MMP-9 and -13 could be used to prime the cartilage for vessel invasion and inhibition of ChM-1 could result in vessel invasion. CTGF inhibition could aid in this vascularization as well as inhibiting chondrocyte maturation. Kartogenin can be used to let mesenchymal cells differentiate towards matrix producing chondrocytes but will also increase the expression of TIMPs, which will interfere with vascularization. Differentiation can also be achieved by viral gene transfer of BMP-2 and -4, but cannot yet be directed to a specific site.
Using embryonic development and vascularization of the cartilage to repair defects will be a complex procedure due to the number of factors involved and the side effects like OA or terminal differentiation and subsequent bone formation. With either embryonic development or resident chondrocytes it will require an off-switch of chondrocyte differentiation. It has to be determined which factor has to be switched on or off at what time. Furthermore, delivery of the specific factors has to be directed to the defect site in order for the right actions without systemic reactions.
The complexity of repairing cartilage defects using embryonic development requires more research to create a complete overview of which factors have to be influenced at which time.

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