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Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation

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1. ABSTRACT

Articular cartilage disorders and injuries often result in life long chronic pain and compromised quality of life, thus regeneration of articular cartilage is a persistent challenge to medical science. One of the most promising therapeutic approaches is cell based tissue engineering which provides a healthy population of cells to the injured site and requires differentiated chondrocytes from the uninjured site as base material. Use of healthy chondrocytes has several limitations and an excellent alternative cell population could be adult marrow stromal cells/mesenchymal stem cells (MSCs) which are known to possess extensive proliferation potential and proven capability to differentiate into chondrocytes. Both, *in vivo* and *in vitro* pliability of MSCs and chondrocytes greatly depends on their microenvironment. Gene and protein expression profiles of both the cell types can be altered by soluble factors from surrounding tissue/ cells or by direct cellular contact. For MSC or chondrocyte-based cartilage repair, inhibition of hypertrophy and stabilization of the cartilaginous phenotype in the implant is a prerequisite for success and long lasting vitality of the repaired tissue.

2. INTRODUCTION

Articular cartilage is an avascular, aneural and highly specialized connective tissue of mesenchymal lineage which mainly functions as a load bearer for joint surfaces. Structurally it comprises chondrocytes embedded in their self contrived highly collagenous and proteoglycan-rich extracellular matrix (ECM). Local chondral lesions of more than 5 mm in diameter do not spontaneously heal, the exact reasons for this shortcoming are yet unknown. However, it is very likely that avascularity of the tissue leads to deficiency of chondroprogenitor cells required for rejuvenation. This theory is backed up by the observation that an osteochondral injury which penetrates through the vascularized subchondral bone does invoke basic repair processes, however the resultant cartilage is mostly fibrous and more rigid than the hyaline matrix. Therapies for damaged cartilage are limited to short term pain relief, inflammation control and surgical intervention. In extreme cases the affected tissue is surgically replaced by synthetic or biological grafts as in autologous osteochondral transfer (OCT). All of the surgical methods have limitations and high risk potential to the donor and recipient tissue (1). In use since 1994, autologous chondrocyte

Table 1. Antigenic profile of MSCs derived from bone marrow and other mesenchymal tissues

Antigen	Bm	Sy	Po	Sm	Ad
STRO-1	++	++	++	+	+
NGFR	+	+	+	++	+
CD 10	+	+	++	+	++
CD29	++	?	?	?	?
CD 44	++	++	++	++	++
CD 49a	++	?	?	?	?
CD 54	+	+	+	+	+
CD 71	++	?	?	?	?
CD 90	++	++	++	++	++
CD 105	++	++	++	++	++
CD 106	++	++	+	+	+
CD 147	++	++	++	++	++
CD 166	++	++	++	+	++
CD 14	-	-	-	-	-
CD 34	-	-	-	+/-	-
CD 45	-	-	-	-	-
CD 117	-	-	-	-	-
VEGFR2	-	-	++	-	-

Bm: Bone marrow; Sy: Synovium; Po: Periosteum; Sm: Skeletal muscle; Ad: Adipose tissue, ++ High expressed (100%); + Low expressed (up to 50%); Not expressed (0-2%), (6-8;61-67)

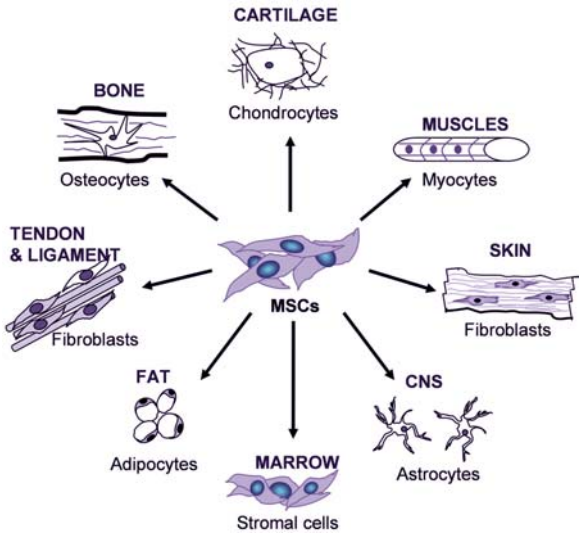

Figure 1. Multi-lineage potential of adult mesenchymal stem cells. Mesenchymal stem cells which are present in adult bone marrow are multipotent and have an extensive proliferation capacity. They can differentiate into multiple lineages as osteocytes, chondrocytes, fibroblasts, adipocytes, astrocytes and myocytes.

transplantation (ACT) is the only FDA (US Food and Drug Administration) approved cell based therapeutic approach to regenerate articular cartilage (2). It provides a healthy population of cells to the injured site by using differentiated chondrocytes from uninjured sites as base material. Its main limitation is that the use of healthy chondrocytes often leads to donor site morbidity. Moreover, differentiated chondrocytes do not proliferate *in vitro.* Attempts to induce proliferation leads to dedifferentiation towards fibroblast-like cells and generates rigid fibrous cartilage where more flexible hyaline cartilage is required. A substitute for healthy chondrocytes could be adult chondroprogenitor marrow stromal cells/ mesenchymal stem cells (MSCs). A major advantage of MSCs for cartilage tissue engineering is their non-immunogeneity which allows use of allogenic cells.

By using MSCs chondrocyte dedifferentiation and limited cell number could be overcome but achievement and maintenance of the articular hyaline phenotype still remains a critical challenge. The process of chondrogenesis can be mimicked *in vitro* by inducing chondroprogenitor stem cells to undergo chondrogenesis through exposure of favourable mechanical and nutritional conditions. For successful *in vitro* regeneration of articular cartilage it is imperative to better understand *in vivo* chondrogenesis during embryonic development and adult repair processes. In this review we will reiterate sources of chondroprogenitor cells and then comprehensively analyze the microenvironmental signals and their prospective effects on chondrogenesis and maintenance of a stable chondrogenic phenotype.

3. SOURCES OF PROGENITOR CELLS

Unlike most of the tissues cartilage consists only of one cell type, the chondrocytes and does not contain sufficient progenitor cells. Hence, the best choice of cells to study chondrogenic differentiation are embryonic stem cells (ESCs) which are totipotent, have unlimited proliferation potential and can be induced to differentiate towards the chondrogenic phenotype among others. However, for various practical and ethical reasons use of ESCs is not always an option for many labs.

3.1. *In vitro* characterization of MSCs

In the early 1970s *Friedenstein et al.* detected multipotent adult MSCs possessing properties of ESCs in bone marrow (3). This early finding was later popularised by *Pittenger et al*. and since then MSCs have been discovered in a multitude of adult tissues (4). Regardless of the source of origin, MSCs possess three distinctive characteristics; they can be expanded *in vitro*, they have an extensive proliferation capacity and they can differentiate into multiple lineages namely, osteocytes, chondrocytes, adipocytes, astrocytes and myocytes (Figure 1) (1;4;5). Until to date no there is conclusive proof of *in vivo* presence of MSCs. The lack of a unique antigenic marker is still the major limiting factor for unambiguously defining MSCs *in vivo* as well as *in vitro* (Table 1). One of the earliest set standards for identification of MSCs is the colony forming unit-fibroblast assay (CFU-F). This assay depends on the adherence of MSCs to tissue culture plastic vessels with spindle-shaped cell colonies arising from a single proliferating cell. Despite the fact that it accounts for an *in vitro* characteristic, the CFU-F assay is still the most relevant assay for identification of MSC populations, in addition to determination of their multilineage potential.

3.2. Tissue sources of MSCs

In addition to bone marrow MSCs have been found in most of the solid mesenchymal tissues. Adipose derived MSCs have been quite extensively scrutinized for their osteo/chondrogenic potential but the original location

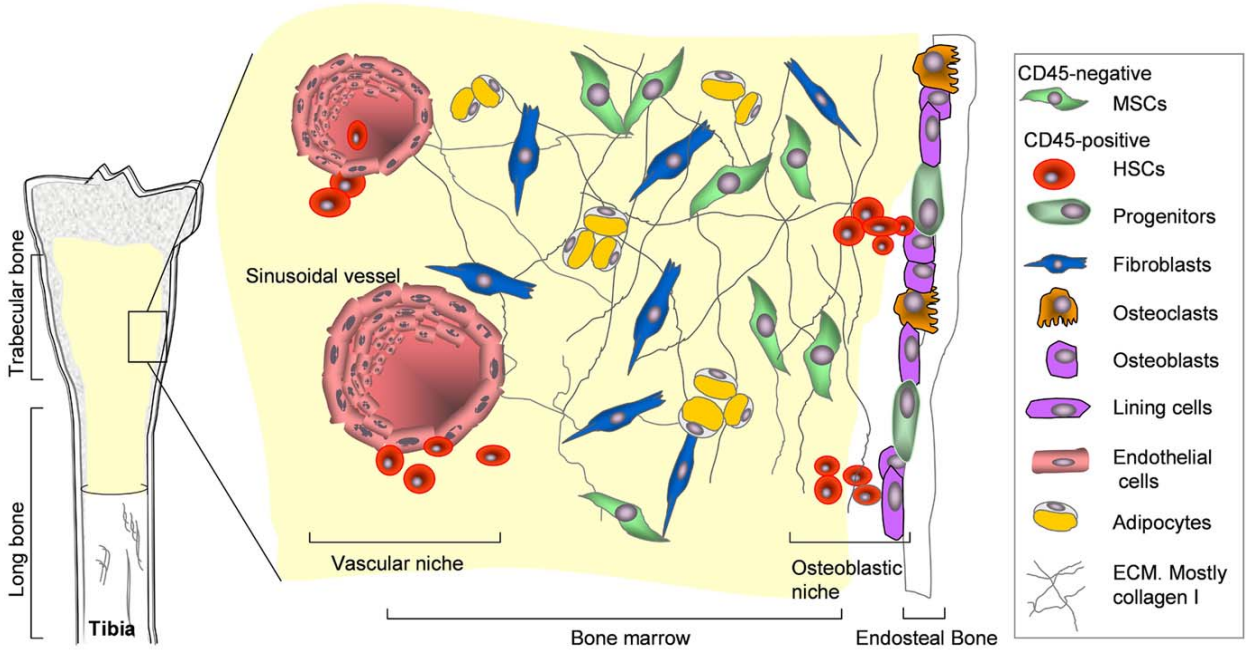

Figure 2. The stem cell niches in bone marrow. In the bone marrow HSCs and their progeny populate the vascular niche which is surrounded by stromal cells derived from MSCs. Naïve MSCs with true stem cell attributes are part of the stroma while MSCs which are committed osteoprogenitor cells reside in the osteoblastic niche. Modified from (12).

of the tissue appears to play a significant role in determining their differentiation potential. Adipose derived MSCs have a higher adipogenic potential while cells from the periosteum exhibit a superior osteogenic and chondrogenic ability (6)**.** Due to their proximity to the bone and the heterogeneity of the cell population it is not surprising that periosteal MSCs exhibit high osteogenic potential while also exhibiting chondrogenic and myogenic capacity (7). Though skeletal muscle derived MSCs are known for their relatively low potential for chondrogenesis they do possess multi-differentiation capacity. Synovial membrane forms the lining of the chondyle surface and it is the most proximal vascularized tissue to cartilage. MSCs derived from the synovial membrane and synovial fluid show high chondrogenic potential which is comparable to that of bone marrow derived MSCs. It is assumed that these cells originate from the bone marrow and migrate to the synovium via vasculature (8). Interestingly, some studies have also indicated the presence of MSC - like progenitor cells in the surface zone of normal and osteoarthritic adult human articular cartilage as well as in immature bovine articular cartilage (9;10). This observation is intriguing because it shows lack of regeneration of diseased articular cartilage in apparent presence of chondroprogenitor cells. A probable explanation is that the MSCs found in cartilage are actually recruited from the synovial membrane as a reparative response to damage. This could also explain the detection of a higher number of MSCs in osteoarthritic (OA) cartilage compared to healthy cartilage. However, the increased frequency of progenitor cells in OA-cartilage could also result from proliferation of resident progenitor cells (10). These observations provoke the presumption that the mere presence of MSCs at the site of injury is not sufficient for induction of repair processes. Whether MSCs require cues from the microenvironment to differentiate towards chondrocytes still needs to be addressed.

4. *IN VIVO* MICROENVIRONMENT OF BONE MARROW DERIVED MSCs

4.1. Osteoblastic and haematopoietic stem cell niches

The cavity of mammalian bone is filled with blood vessels and soft bone marrow (BM) and is the only organ so far identified which is host of two types of functionally cooperating stem cells. The main population consists of haematopoietic stem cells (HSCs) which is supported by bone marrow stroma containing a small population of non-blood forming MSCs. In the stroma or bone marrow microenvironment, MSCs coexist with endothelial cells, macrophages, adipocytes, fibroblasts, osteoprogenitor cells and HSCs and their progeny (11); (12). The stem cell niche *in toto* is defined as where stem cells reside and undergo self-renewal and/ or differentiation; the niche reserved for MSCs in the marrow is not well explored. However, existence of two distinct stem cell niches in the BM has been well argued; an osteoblastic niche for osteoprogenitor cells and a vascular niche for HSCs where the mature haematopoietic cells are released into the vascular system. The HSC niche has been known since 1978 and the role of their physiological microenvironment both, as structural support and mediator of cell signalling has been studied in depth unlike the osteoblastic niche. MSCs exist in different commitment and differentiation states, most likely the so called naïve MSCs with true stem cell attributes reside as part of the stroma, but the MSCs with committed osteoblastic progenitor status reside in the osteoblastic niche (Figure 2) (13). Both, *in vivo* and *in vitro* plasticity of MSCs greatly depends on the

microenvironment. It has been convincingly shown that the scale of heterogeneity of the extracted cell population determines their differentiation potential. Historically, MSCs as part of BM stroma have been shown to support haematopoiesis even before their mesodermal progenitor cell status was established (14). Now it is also known that removal of the native soluble and cell-contact signaling network of the bone marrow reduces plasticity and proliferation capacity of MSCs *in vitro*. Such discoveries indicate that the signalling cues, cytokines and growth factors from the cellular microenvironment are vital for differentiation, proliferation and maintenance of the differentiated status of HSCs and MSCs (15). An interesting example is the chemokine, stromal derived factor 1 (SDF-1) which is involved in homing of HSCs in bone marrow. By expressing SDF-1 MSCs may affect homing and growth of HSCs, a new finding that indicates the presence of SDF-1 receptor not only on HSCs but also on MSCs. *Kortesidis et al.* have indicated a role of the SDF-1 in maintaining osteogenic capability of MSCs. This can be taken as an example of a mutual and reciprocal communication between HSCs and MSCs in the maintenance of bone marrow integrity (16).

4.2. CD45-positive cells enhance chondrogenic gene expression in MSCs

In our earlier *in vitro* studies we have shown persistent presence and attachment of CD45-positive predominantly non-adherent haematopoietic precursor cells among adherent adult MSCs when no specific surface antigen based selection is carried out. Other groups support this observation by showing that adherence to tissue culture plastic alone is a weak discrimination criterion for isolating a homogeneous MSC population, because neural cells, monocytes and macrophages also adhere to plastic surfaces (17-19). The persistent presence of CD45-positive haematopoietic cells and the variation of their proportion in primary cultures of MSCs could also explain the occurrence of extensive variations usually seen in differentiation experiments with bone marrow derived cells.

Our observation that the CD45-positive cells exert a positive impact on chondrogenic differentiation of MSCs indicates that haematopoietic cells create a microenvironment that may enhance expression of chondrogenic – associated marker genes in MSCs. We observed induction of master transcription factor Sox9 and a profound increase in gene expression of collagen II, COMP and aggrecan; latter are structural components of an extracellular matrix highly specific for hyaline cartilage. On the other hand, genes normally associated with osteogenesis were also upregulated, e.g. collagen I and hypertrophic differentiation indicator collagen X. Runx2, a transcriptional activator for osteoblastic differentiation during the process of endochondral ossification remained largely unaltered. These data indicate a pro-differentiation microenvironment promoting differentiation of MSCs towards the chondrogenic lineage by up regulating specific genes. However, the observed gene expression profile indicates an early push on chondrogenically differentiating MSCs to enter the pre-hypertrophic / hypertrophic differentiation cascade and to proceed towards terminal differentiation (20). Possibly, neighbouring CD45-positive cells in this setting are pre-programmed to promote endochondral ossification instead of a stabilized chondrogenic phenotype required for obtaining permanent cartilage. CD45 which is a negative regulator of adhesion plays a role in bone remodelling indicated by increased osteoclast activity in CD45 knockout mice (21).

The heterotrophic nature of bone marrow derived cells suggest that *in vivo* interactions of different cell types are very likely and should be addressed accordingly. Based on our observations we speculate that the stromal CD45-positive population *in toto* is responsible for regulation of gene expression. Identification and isolation of factors that are capable of modulating genes involved in differentiation and dedifferentiation processes of MSCs and chondrocytes will allow us to manipulate the chondrogenic phenotype for *in vitro* engineering of cartilage tissue.

5. *IN VIVO* CHONDROGENIC INDUCTION VIA MICROENVIRONMENT

During skeletal development chondrogenesis is described as the process that results in the formation of the cartilage intermediate (Anlagen), which eventually leads to bone formation by endochondral ossification. Cartilage Anlagen therefore constitute the primary skeleton of the embryo and are vital for pattern formation and longitudinal growth of the whole body. Permanent cartilage structures which persist throughout life are joints, ears and airways. The process of chondrogenesis begins when mesenchymal cells form condensations which prefigure the future skeletal elements and are regulated by mesenchymal – epithelial cell interactions. Once committed, chondroprogenitor cells continue to differentiate towards generation of embryonic chondroblasts which turn into chondrocytes after birth. These chondrocytes become arranged in a typical pattern in growth plates where they start to proliferate and proceed through a pre-hypertrophic and hypertrophic stage until they finally undergo terminal differentiation and apoptosis to allow bone formation. This process is regulated by numerous growth factors, transcription factors and proteases. Their comprehensive coordinated expression pattern and mode of action remains yet to be satisfactorily unravelled (Figure 3) (22-27).

Developmental processes are usually terminated when adulthood is reached, however some regenerative processes may become reinitiated as a consequence of injury. Specific tissues have the ability to recapitulate stages of initial development to regenerate. An excellent example is the repair of bone fractures which is considered a process in adults where new bone is generated instead of scar tissue. In case of motion at the site of injury, the primary mode of skeletal repair is through the formation of a cartilaginous scaffold or callus, which is gradually replaced by bone. The cellular and molecular processes that contribute to bone regeneration after fracture have many similar features to those which occur during embryonic and postnatal endochondral ossification (28). Compared to embryonic and postnatal epiphyseal growth, fracture repair uses a more primitive mechanism to achieve rapid growth

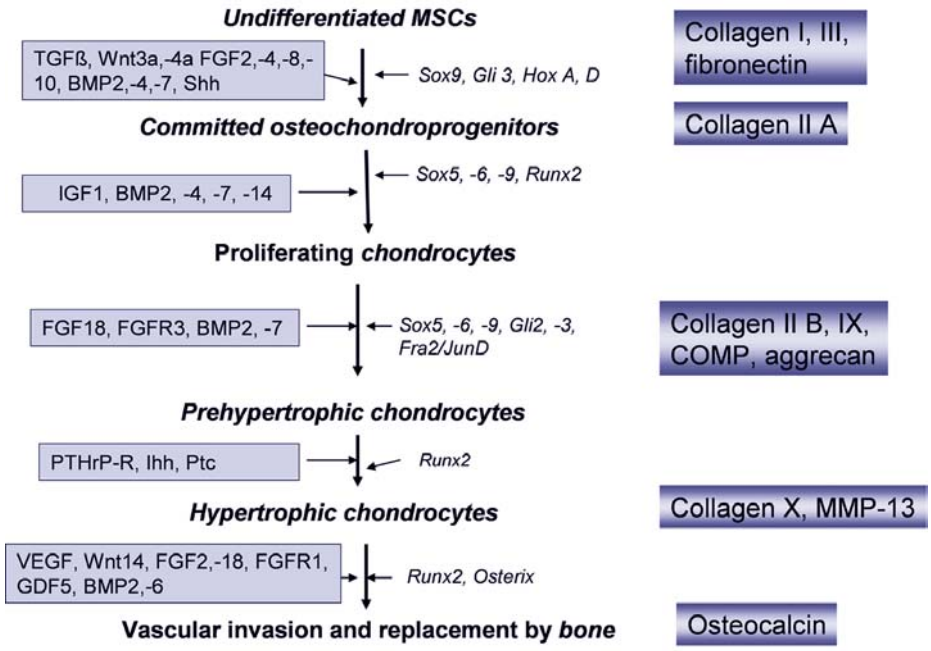

Figure 3. Chondrogenic differentiation cascade. Mesenchymal stem cells undergo chondrogenic differentiation during embryonic development and produce cartilaginous Anlagen for bone development. The entire cascade is governed by an interconnected web of growth factors, transcription factors, matrix molecules etc. The highest profile among them has transcription factor Sox9 whose expression is necessary for the initiation of chondrogenic differentiation while at hypertrophic stages it is suppressed. This figure shows different stages of chondrogenesis along with the stage-specific ECM markers. Signaling and growth factors are shown in light grey boxes and matrix molecules are depicted in two-coloured grey boxes (based on 23, 58-60).

needed for the regenerative process. Such differences may also arise from the differing functional role of the fractured callus in stabilizing the fracture site plus providing a template for new bone formation. This basic difference may provide the tool for identification of regulatory mechanisms which control chondrocyte proliferation, cellular volume and matrix deposition in adults (29).

6. EFFECT OF CHONDROCYTE DERIVED SOLUBLE FACTORS ON DIFFERENTIATED CELLS *IN VITRO*

Cartilage is a paracrine organ known to influence proliferation and differentiation of neighbouring tissues and cells. Embryonic epiphyseal longitudinal bone growth is critically dependent on specific signals from the cartilage proper itself. The same is observed in adults during fracture repair. To identify and study specific factors responsible for such communication, clearly defined *in vitro* cell culture models are required. Some studies have addressed this issue by coculturing chondrocytes from articular cartilage with osteoblasts, endothelial cells, macrophages or growth plate chondrocytes (Figure 4A).

It is known that chondrocytes of normal articular cartilage do not undergo terminal differentiation whereas growth plate chondrocytes do. By coculturing articular and growth plate chondrocytes in a set up which restricted interaction to paracrine communication, *Jikko et al.* have demonstrated that soluble paracrine factors alone are responsible for alteration of the chondrogenic phenotype. Articular chondrocytes were able to suppress matrix calcification and alkaline phosphatase activity in cocultured growth plate chondrocytes, indicating that articular chondrocytes produce factors which inhibit or delay terminal differentiation (30). One likely candidate responsible for inhibition of terminal differentiation is TGF-beta. Coculturing of chondrocytes isolated from the sternum of chick embryos either with articular chondrocytes or with conditioned medium from articular chondrocytes induces inhibition of collagen X expression in sternal growth plate chondrocytes. However, treatment with an antiserum against TGF-beta did not neutralize inhibition of collagen X indicating that TGF-beta alone is not responsible for regulation of terminal differentiation (31). Surely other, yet unidentified factors are also involved which are either redundant or work in cooperation with TGF-beta. During endochondral ossification, cartilage maturation and conversion of hypertrophic cartilage to bone is delayed by paracrine signals derived from periosteal / perichondrial tissue or from the cartilage proper itself. Formation of new blood vessels and their penetration into hypertrophic cartilage appears to be essential for the elimination of this developmental barrier. Consistent with this notion, the terminal differentiation barrier in chondrocytes from the caudal part of 17-day old chick embryo sternum can be eliminated by coculturing them with endothelial cells *in vitro*. The humoral activity secreted by endothelial cells consists of proteinases sufficient to break down the terminal differentiation barrier

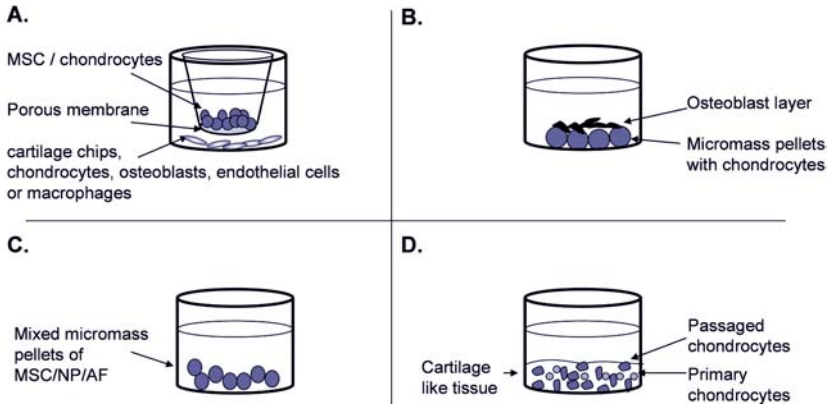

Figure 4. Schematic representation of coculture systems. A. MSCs or chondrocytes kept in alginate beads or agarose were cocultured with either endothelial cells, growth plate chondrocytes, osteoblasts, macrophages or cartilage explants. The cocultures were separated by a 1µm porous membrane allowing paracrine contact only. B. An osteoblastic monolayer was seeded onto high density micromass pellets consisting of chondrocytes allowing direct cell-to-cell contact. C. MSCs were cocultured with nucleus pulposus (NP) or annulus fibrosis (AF) cells in a micromass pellet system allowing direct cell-to-cell contact. Cells were seeded in different ratios. D. Passaged, dedifferentiated chondrocytes were cocultured with primary, differentiated chondrocytes in a monolayer setting. According to the ratio of differentiated versus dedifferentiated chondrocytes cartilage-like tissue was generated.

inherent to this chondrocyte population (32-34). Fibril degradation is mainly mediated by matrix metalloproteases and, among these, MMP-9 assumes an outstanding role. Besides further degrading collagen II fragments (gelatine), it also directly degrades collagen XI. Latter constitutes the most critical step of fibril breakdown because it disrupts the macromolecular organisation and stability of cartilage fibrils. Notably, conversion of pro-MMP-9 into the active form can be achieved by chondrocytes from osteoarthritic but not from healthy cartilage. In order to identify the responsible factors and the activation cascade OA-chondrocytes have been cocultured with macrophages known to secrete pro-MMP-9 without activating the enzyme. It was shown that the stepwise activation process of pro-MMP-9 involves MMP-13, secreted from OA-chondrocytes and MT1-MMP, a membrane bound MMP, also expressed by chondrocytes which converts pro-MMP-13 to its active form. This scenario clearly demonstrated that chondrocytes actively participate in collagen matrix degradation and, thus contribute to the initiation or even maintenance of osteoarthritis (35-37). In OA articular chondrocytes undergo dramatic changes in gene expression induced by changes in the surrounding matrix, mechanical strain and alterations in supply with growth factors and cytokines. However, osteoblasts from the underlying subchondral bone can also induce phenotypic shifts in OA-chondrocytes. Coculturing of OA-chondrocytes with OA-osteoblasts from the subchondral bone induces inhibition of aggrecan production and a concomitant significant increase in MMP-13 synthesis. The cocultured osteoblasts also decrease Col2a1, Sox9 and PTHrP / PTH-receptor gene expression in the chondrocytes whereas pleiotrophin (OSF-1) mRNA level was increased. OSF-1 is normally restricted to growth plate cartilage, but becomes re-expressed in OA-chondrocytes. These metabolic changes could be considered as a phenotypic shift towards the hypertrophic stage. This study has also excluded TGF-beta as the sole mediator of these gene expression changes opening the search for other yet unidentified molecules needed to mediate the fate of chondrocytes either towards permanent or hypertrophic cartilage (38-40).

7. PARACRINE CROSSTALK BETWEEN CHONDROCYTES AND MSCs AFFECTS EXPRESSION OF CHONDROGENIC MARKERS

7.1. *In vitro* coculture systems

For long-term repair and regeneration of focal cartilage defects chondrocytes have been implanted at the site of injury, however, not much attention has been paid to microenvironmental effects. Information regarding mutual paracrine and direct cellular effects of surrounding native tissue and newly transplanted cells is scarce. To mimic the physiological conditions at the injured cartilage site after a stem cell transplant, we and others have setup *in vitro* systems for the coculture of MSCs or ESCs with cartilage tissue explants or chondrocytes (Figure 4A). These systems aim to detect effects on molecular control of chondrogenesis exerted by soluble signaling factors originating from the cartilage tissue or the chondrocytes. Such *in vitro* studies provide precious information regarding molecular control, which is of direct relevance to future clinical applications of stem cell-based regenerative medicine. The above mentioned coculture systems were designed according to the hypothesis that growth factors such as TGF-beta or BMPs released by the cartilage tissue may compensate for external growth factors required to stimulate chondrogenesis in ESCs or MSCs. Both cell types were cultured in vessels which separated them from the cartilage tissue explant by a 1µm thick porous membrane allowing paracrine contact only. Coculturing of a human ESC line maintained in suspension with human chondrocytes in the presence of 10% FBS resulted in induction of collagen II and Sox9 protein expression by paracrine factors derived from the chondrocytes (41). In our MSC–cartilage explant system, the MSCs were

suspended in 1.2% alginate which provides a high density three-dimensional chondrogenic favorable environment. Unlike other studies this culture system is carried out in serum free chondrogenic medium in absence of externally added growth factors (Ahmed et al., Soluble signaling factors derived from differentiated cartilage affect chondrogenic differentiation of rat adult marrow stromal cells; in press Cell. Physiol. Biochem.).

7.2. Cartilage influences gene expression and biosynthesis of chondrogenic factors in MSCs

In this system, we have shown early onset of chondrogenesis indicated by premature upregulation of Sox9 gene expression. Several signaling pathways are involved in regulating Sox9 and its downstream genes during cartilage differentiation. One of them, the TGF-beta cascade, induces Sox9 gene expression and transcriptional activity through Smad3 (42) indicating that TGF-beta, released from cartilage explants in the coculture system, possibly accounts in part for the observed increase in Sox9 gene expression. On the other hand, VEGF has been described to suppress Sox9 mRNA levels during treatment of chick embryo limb buds (43). In the coculture supernatant we have detected high level of VEGF which may have induced suppression of Sox9 in MSCs, thereby counteracting the influence of TGF-beta. Despite suppression of Sox9 mRNA gene expression of Col2a1 remains up regulated temporarily. This observation indicates that although Sox9 is imperative for initiation of chondrogenesis, an active Sox9 gene above basal level of un-induced MSCs might not be mandatory in later stages of chondrogenic differentiation.

The most profound effect of cartilage-derived factors was observed on suppression of collagen X gene expression and protein secretion. From day 21 on monocultured MSCs release high amounts of collagen X into the culture supernatant, while cocultures with cartilage explants do not. Suppression of collagen X secretion in the presence of cartilage depicts delayed hypertrophy. We assume that cartilage derived soluble factors have the ability to profoundly reduce collagen X secretion. Furthermore, absence of collagen X secretion is presumably due to suppression at the biosynthesis level. Our observation of a profound increase in pro-MMP-13 secretion in coculture and single-cultured cartilage explants suggests an interesting explanation. MMP-13 is known to degrade collagen X (44) however, lack of collagen X in supernatant of cocultured MSCs can not be due to MMP activities because no collagen X degradation products or reduced collagen X signal intensities were detected in the cultures. In MMP-13 knockout mice *Inada et al.* have demonstrated a remarkable enlargement of the anatomic domain where collagen X is expressed by hypertrophic growth plate chondrocytes and an increase of collagen X deposition in this domain. It appears that in addition to proteolytic activity, MMP-13 might exert a yet unknown regulatory impact on collagen X biosynthesis (45;46). Therefore, suppression of collagen X gene expression and accordingly biosynthesis in cocultures might be rather due to a regulatory effect of MMP-13 than to a proteolytic effect (Ahmed et al., Soluble signaling factors derived from differentiated cartilage affect chondrogenic differentiation of rat adult marrow stromal cells; in press Cell. Physiol. Biochem.).

Another key player responsible for collagen X regulation might be VEGF as it is one of the most important mediators of angiogenesis. VEGF is abundantly expressed during embryogenesis but in adults it is only found in restricted tissues and cell types, as in osteoarthritic chondrocytes, but not in resting chondrocytes (47;48). Interestingly, we have detected VEGF-164 alpha in increasing amounts in cocultures and single-cultured cartilage explants, where the main source seems to be cocultured MSCs (Ahmed et al., Soluble signaling factors derived from differentiated cartilage affect chondrogenic differentiation of rat adult marrow stromal cells; in press Cell. Physiol. Biochem.). VEGF is known to stimulate receptor phosphorylation of its VEGF-receptors 1 and -2 (Flt-1/ Flk-1-KDR), thereby activating the mitogen activated protein kinases ERK/1/2 which induce long-lasting activation of the transcription factor AP-1. As a consensus site for AP-1 has been identified within the promoter of collagen X, VEGF binding to its receptor (VEGFR1) might contribute to suppression of gene expression of collagen X, while at the same time it is known to induce secretion of MMP-13 (Figure 5) (49).

8. MODULATION OF CELLULAR PHENOTYPE BY DIRECT CELL-TO-CELL CONTACT

All studies listed so far described culture systems which were not based on direct physical contact but separated in a way which allowed communication only via diffusion of secreted soluble factors. Besides paracrine factors direct cellular contact and interactions between cells and tissues are believed to mutually affect differentiation and proliferation status of the cocultured cells. *In vitro* coculturing of MSCs with differentiated cells, as chondrocytes, osteoblasts or nucleus pulposus cells could be a reliable method to generate a sufficient number of pre-differentiated cells to be used in cell–based therapies for regeneration of destroyed articular cartilage surfaces in OA, lesions produced by chondral trauma or degenerated intervertebral discs.

8.1. Coculture models allowing cell-to-cell contact

Jiang and colleagues exploited a coculture model where an osteoblastic cell monolayer was directly seeded onto a high density micromass pellet consisting of chondrocytes (Figure 4B). In their model chondrocytes increased collagen II production and reduced glycosaminoglycan (GAG) synthesis while osteoblasts showed delayed mineralization and increased collagen I production. Whether coculture has an effect on collagen X production and thereafter on hypertrophic differentiation remains to be elucidated (50). *Richardson et al.* cocultured nucleus pulposus (NP) cells with MSCs using a monolayer coculture model which allows direct cell-to-cell contact (as in figure 4D). Their system shows intriguing results suggesting a mutual influence of both cell types. They have demonstrated a profound increase of Sox9, collagen II and aggrecan gene expression rates in MSCs only after 7 days

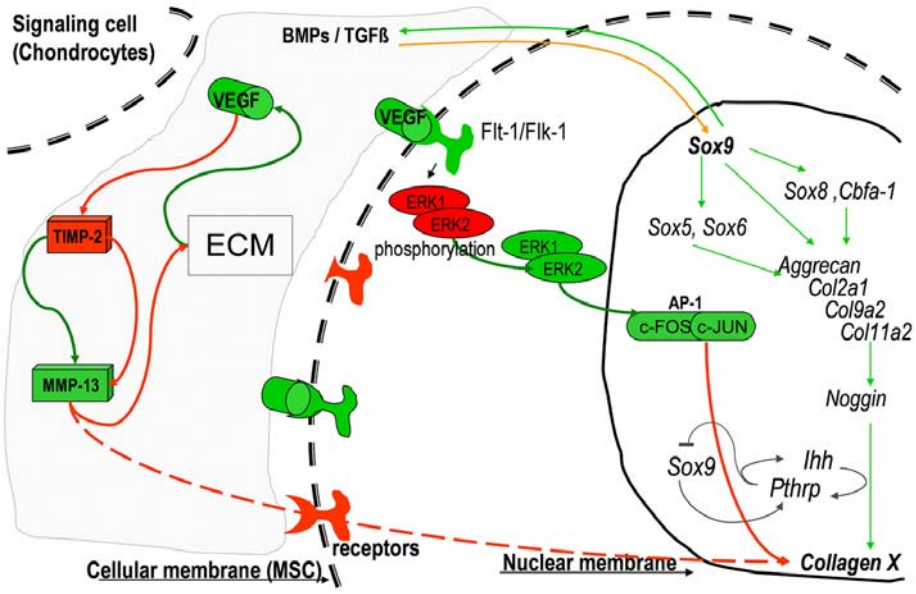

Figure 5. Molecular events during chondrogenesis. Schematic representation of known and putative molecular control of chondrogenic differentiation by selected signaling molecules. The effect can be pre-transcriptional by suppressing gene expression, post-transcriptional on protein biosynthesis or post-translational by matrix modulation via proteases and their inhibitors. Green arrows indicate upregulation of gene and protein expression, yellow arrows depict a positive effect to maintain the expression and red arrows mean inhibition of the gene and protein expression.

of direct coculture with NP cells with the highest induction rates observed when the NP cell to MSC ratio was 3:1. Coculture without contact or coculture of MSCs with human dermal fibroblasts did not produce any significant changes in matrix gene expression rates (51). Notably, cellular origin has a profound impact on differentiation response. In micromass pellet based cocultures of MSCs with annulus fibrosis (AF) cells glycosaminoglycan production rate was significantly higher compared to cocultures with NP cells. This coculture system was formed by addition of MSCs to disc cells in a 1:1 ratio, however, which of the cells contributed to the GAG increase is not discernible in this experimental set up (Figure 4C) (52). MSCs themselves are capable of increasing the viability and proliferation rate of NP cells in a direct cell-to-cell contact coculture system while at the same time enhancing secretion of TGF-beta, IGF-1, EGF and PDGF from NP cells (53).

The prospect of implanting undifferentiated or dedifferentiated cells into cartilage defects without time consuming and cost effective *ex vivo* pre-differentiation steps appears very intriguing. Passaging chondrocytes to increase cell number is a common way to overcome the major limitation of cartilage tissue engineering, however, *in vitro* expansion results in dedifferentiation of the cells. Interestingly, the coculture of passaged and non-passaged chondrocytes (differentiated chondrocytes) resulted in the formation of hyaline-like cartilage matrix *in vitro* (Figure 4D). While the presence of as few as 5% primary chondrocytes was sufficient to induce this response, significant accumulation of a cartilaginous matrix was detected when 20% - 40% of the total number of cells was differentiated (54). The exact mechanism by which the differentiated and dedifferentiated chondrocytes communicate is unknown. One possible mode of action could be cross talk between cells via gap junctions which has been observed in coculture of osteoprogenitor cells with endothelial cells where osteogenic differentiation was induced through the gap junction protein connexin 43 (55).

8.2. Combined influence of paracrine and cell-to-cell contact on differentiation of MSCs

Although not many studies have been conducted to analyse consequences of direct cell-to-cell contact between differentiated and undifferentiated cells and tissues, the results thus far obtained are promising. They clearly demonstrate a yet undefined, nevertheless, profound combined influence on matrix gene expression, protein production and growth factor release which is superior to a solely paracrine contact between different types of cells and tissues. Of course, connecting the effects to specific soluble signaling molecules bound to cell surface receptors or to signaling from direct cell-to-cell contact remains a challenge for future investigations. However, this novel methodology has obvious implications for the future of cell-based tissue engineering for cartilage repair. The data implies that creating a specific microenvironment can induce various differentiation pathways in multipotent, mostly undifferentiated MSCs guided by the neighbouring differentiated cell type. Further manipulation of the humoral and cellular microenvironment might help omit time-consuming pre-implantation differentiation of MSCs or expansion resulting in dedifferentiation of chondrocytes in cell culture. In the light of these *in vitro* studies, it is presumable that the surrounding cartilage tissue would be

sufficient to induce chondrogenic differentiation of cells and program them to produce a cartilaginous matrix resembling the original template.

9. SUMMARY AND PERSPECTIVES

Arguably one of the most challenging complex tissues for regenerative medicine is articular cartilage. It's avascular nature, dense extracellular matrix, limited number of cells, and absence of clearly defined progenitor cells are but a few issues (1;56). For *ex vivo* cartilage regeneration multipotent adult somatic MSCs are the tool of choice because of their *in vitro* pliability and retention of differentiation potential. In addition, for cell based cartilage therapy use of MSCs instead of chondrocytes as a cell source also avoids donor site morbidity (57). However, production of tissue engineered functional cartilage with articular characteristics is still a challenge. To effectively address this issue, employment of two elements of tissue engineering, the microenvironmental signals and the cells, needs to be optimized. Thus, studies aiming at better understanding of the chondrogenic differentiation pathways in adults for tissue regeneration, the molecular regulatory mechanisms and the modulating influence of neighboring cells are essential. Studies on paracrine signals from the microenvironment and their effect on MSC commitment and differentiation have demonstrated that the chondrogenic differentiation pathway of bone marrow derived MSCs is strongly influenced by the representative microenvironment. The important observation that cartilage tissue derived factors can suppress collagen X expression is a foundation for future studies on soluble factors to discover a direct correlation between the effect and the putative effectors. Models employing direct cell-to-cell contact between differentiated and undifferentiated cells reveal promising data indicating a stronger influence as solely paracrine interactions. Nevertheless, identification of signaling molecules and cascades responsible for cell fate determination and the identification of the nature of interaction whether paracrine, cell contact dependent or combined are empirical for successful future cartilage regenerative medicine.

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Abbreviations: MSCs: mesenchymal stem cells, HSCs: haematopoietic stem cells, ECM: extracellular matrix, OCT: osteochondral transfer, ACT: autologous chondrocyte transplantation, ESCs: embryonic stem cells, OA: osteoarthritis, BM: bone marrow, MMP: matrix metalloproteases, VEGF: vascular endothelial growth factor, BMP: bone morphogenetic protein, GAG: glycosaminoglycan, TGF-beta: transforming growth factor beta, IGF: insulin like growth factor, PDGF: platelet derived growth factor, NP: nucleus pulposus; AF: annulus fibrosis, PTH: parathyroid hormone, PTHrP: parathyroid hormone related peptide, AP: alkaline phosphatase

Key Words: Mesenchymal stem cells, Chondroprogenitor cells, Chondrocytes, Articular cartilage, Microenvironment, Regulatory factors, Coculture, Review

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