AUS DEM LEHRSTUHL FÜR UNFALLCHIRURGIE
DIREKTOR: PROF. DR. DR. VOLKER ALT
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

ATTENUATION OF HYPERTROPHY IN CHONDROGENICALLY DIFFERENTIATING HUMAN MESENCHYMAL STEM CELLS VIA TREATMENT WITH A RETINOIC ACID RECEPTOR INVERSE AGONIST

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
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Zusammenfassung

Die körpereigene Fähigkeit zur Regeneration von Gelenkknorpel nach traumatischer oder degenerativer Schädigung ist stark eingeschränkt. Mesenchymale Stammzellen (MSCs) sind eine vielversprechende Quelle zur Regeneration von mesenchymalem Gewebe wie etwa hyalinem Knorpel. MSCs haben das Potential zukünftig autologe Chondrozyten in der Therapie von Knorpeldefekten zu ersetzen. Es besteht die Chance, dass die pluripotenten Zellen die embryonale Entwicklung von Gelenkknorpelzellen während der Skelettformation nachvollziehen können.

Jedoch exprimieren *in vitro* chondrogen differenzierten MSCs hypertrophe Marker wie Kollagen X und Matrixmetalloproteinase 13 (MMP13). Dies zeigt, dass die Chondrogenese der MSCs nicht auf einer für Gelenkknorpelzellen üblichen Entwicklungsstufe endet, sondern spontan weiterläuft hin zu einem hypertrophen Stadium, wie es für die Chondrozyten der Wachstumsfuge während der enchondralen Ossifikation typisch ist. Da Hypertrophie jedoch langfristig in Apoptose und Ossifikation mündet, schränkt dieser Umstand den Einsatz von MSCs in tissue engineering von Gelenkknorpel maßgeblich ein.

In der Skelettentwicklung übt der Retinsäure - Rezeptor (RAR) – Signalweg einen wichtigen Einfluss auf die Entwicklung von mesenchymalen Vorläuferzellen aus. Während Retinoide die Chondrogenese hemmen und die Hypertrophie in der Wachstumsfuge fördern, scheint die Unterdrückung der RAR-Wirkung für frühe chondrogene Differenzierungsstufen notwendig zu sein. Daher haben wir die Hypothese aufgestellt, dass die Behandlung von chondrogen differenzierenden Chondrozyten mit dem inversen RAR-Agonisten BMS 204,493 die Hypertrophie hemmen kann. Um diese Hypothese zu überprüfen haben wir die Wirkung von BMS auf humane MSCs auf histologischer, genetischer sowie Protein-Ebene untersucht. Hypertrophie wurde in chondrogen vordifferenzierten MSCs durch den Entzug von transforming growth factor β (TGFβ) und Dexamethason sowie durch den Einfluss von bone morphogenic protein 4 (BMP4) induziert. Die Behandlung mit BMS bewirkte nach Einleitung der Hypertrophie eine Hemmung der hypertrophen Differenzierung der hMSCs, deutlich zu erkennen an der geringeren Zellgröße, ALP-Aktivität und Kollagen X - Genexpression sowie - Produktion. Die Wirkung von BMS war abhängig vom Zeitpunkt der Applikation und am stärksten ausgeprägt bei Anwendung in der frühen Phase der Differenzierung. Die Möglichkeit die Entwicklung von MSCs durch die

Hemmung des RAR-Signalwegs durch BMS zu modifizieren dürfte für die Produktion von stabilem Gewebe zur Regeneration von Gelenkknorpel hilfreich sein.

Abstract

Cartilage's potential to regenerate itself after damage has occurred is very limited. Mesenchymal stem cells (MSCs) are a promising source for the regeneration of mesenchymal tissue such as hyaline cartilage. MSCs may have the potential to replace autologous chondrocytes in the therapy of cartilage defects and to provide even better results. Furthermore, there is a chance that they are able to recapitulate the embryonic lineage transitions originally involved in the formation of joint tissue.

In vitro chondrogenically differentiating MSCs have the tendency to undergo hypertrophy mirroring the fate of transient 'chondrocytes' in the growth plate. As hypertrophy would result in ossification this fact limits the use of MSCs for cartilage tissue engineering applications. During limb development retinoic acid receptor (RAR) signaling exerts an important influence on cell fate of mesenchymal progenitors. While retinoids attenuate chondrogenesis and foster hypertrophy in the growth plate, suppression of RAR signaling seems to be required for early chondrogenic differentiation. Therefore, we hypothesized that treatment of chondrogenically differentiating human MSCs (hMSCs) with the RAR inverse agonist, BMS204,493, will attenuate hypertrophy. To test this hypothesis, we analyzed the effect of BMS on hMSCs on histological, genetical and protein level. Hypertrophy was induced in chondrogenic pre-cultured MSC pellets by withdrawal of transforming growth factor β (TGFβ) and dexamethasone and addition of bone morphogenetic protein 4 (BMP4). Upon induction of hypertrophy, BMS treatment reduced hypertrophic conversion in hMSCs, clearly shown by decreased cell size, ALP activity and collagen type X gene expression and deposition. BMS effects were dependent on the time point of application and strongest after early treatment during chondrogenic pre-cultivation. The possibility to modify hypertrophic cartilage via attenuation of RAR signaling by BMS may be helpful in producing stable engineered tissue for cartilage regeneration.

1 Introduction

1.1 Cartilage

Cartilage is a highly specialized connective tissue that fulfills several functions in the human body. Most important is its ability to withstand mechanical load without getting deformed. Cartilage consists of a single type of differentiated cells, the chondrocytes. The chondrocytes form small isogenic cell groups called chondrons, which are surrounded by a rich extracellular matrix (ECM). The ECM is built, maintained and remodeled by the chondrocytes themselves. The ECM mainly contains two components: An organized network of collagen fibers, basically collagen II but also collagen type I, IX and XI, and an amorphous substance of proteoglycans (mostly aggrecan) and glycosaminoglycans (mostly hyaluronan). Aggrecan and hyaluronan form large aggregates and because of their negative loading due to phosphorylation they have the capability to bind water. This results in the pressure elasticity with high resistance to stress and strain and therefore in the inevitable biomechanical properties [1]–[3].

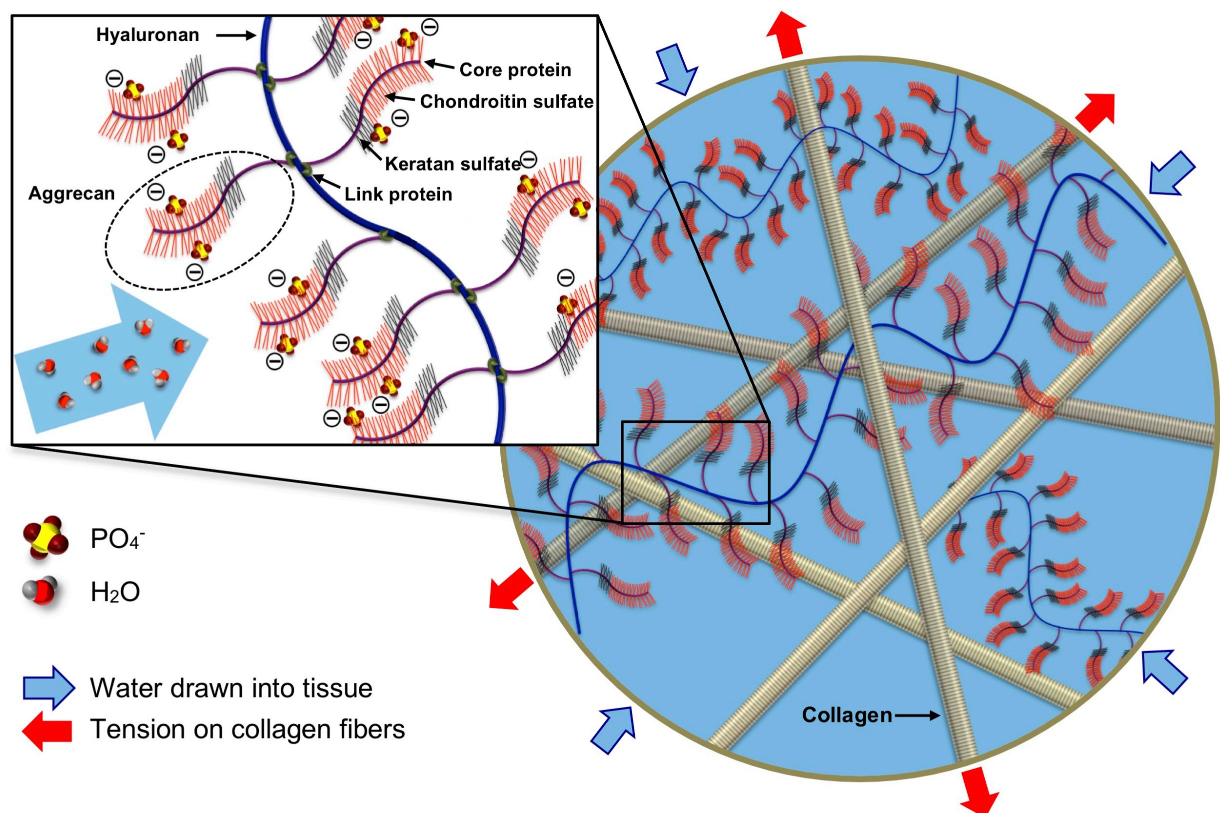


FIGURE 1 **Composition of cartilage ECM: Combined functions of collagen fibers and proteoglycans.** Following the negative load of proteoglycans water flows into the cartilage. Due to the swelling pressure tension is applied on the framework of collagen fibers. The interaction between the structures allows the characteristic biochemical properties of the cartilage

Depending on the kind of cartilage the ECM differs in its composition. There are neither nerves nor lymphatic vessels nor blood vessels in cartilage, so it has to be completely supplied with nutrients by diffusion from the capillaries of the perichondrium or the synovial fluid in the articular cavity. Thus, chondrocytes show just a low metabolic activity and depend primarily on anaerobic metabolism [1]–[3].
Because of the various functional needs three different forms of cartilage have developed:

1.1.1 Elastic cartilage

Elastic cartilage can be found in the external ear, epiglottis and larynx. It adds strength and elasticity to these structures to maintain their shape. Elastic cartilage is surrounded by a perichondrium, a dense connective tissue with an outer fibrous layer of fibroblasts that produce collagenous fibers and an inner chondrogenic layer of undifferentiated progenitor cells that can differentiate to chondroblasts and chondrocytes. The chondrocytes of the elastic cartilage are embedded in an extracellular matrix containing a network of elastic fibers [2]–[5].

1.1.2 Fibrocartilage

The strongest kind of cartilage is the fibrocartilage. It is a mixture between cartilaginous tissue and a fibrous tissue composed of dense collagen fibers. As a specialty it contains also collagen type I apart from collagen type II, which is usually more often to be found in cartilage. Fibrocartilage has no perichondrium. It is part of the intervertebral discs and the joint capsule and forms the junction between tendons and bones [2]–[4].

1.1.3 Hyaline cartilage

The most abundant form of cartilage is the hyaline cartilage. As “hyalos” is the Greek word for glassy, hyaline cartilage has a milky, transparent appearance. It is mainly located in the joints but also in rips and the human airways such as nose, larynx, trachea and bronchi. During enchondral ossification in skeletogenesis and in the growth plate of long bones it acts as a precursor of the bone. As the main parts of the extracellular collagen are fine collagen type II fibers, it’s the weakest kind of cartilage. Beyond the joint cavity hyaline cartilage is surrounded by perichondrium [1]–[4], [6].

1.1.4 Articular cartilage

Articular cartilage is a special form of hyaline cartilage that covers the surface of diarthrodial joints. Its fundamental function is to feature proper conditions for articulation as an interface between two articulating bones, which means after all to reduce the friction to a minimum and to provide biomechanical stability as it acts as a buffer and thus protects the subchondral bone from mechanical impaction. The smooth hyaline cartilage without a perichondrium in combination with the synovia, a fluid rich of hyaluronan, as a lubricant is especially well suited for this job.

Articular cartilage is divided into different zones depending on the structure of the ECM components and on the distinct morphologies and orientation of chondrocytes. Cells in different layers express markers that are characteristic of each zone. The layered architecture of the articular cartilage is the result of mechanical forces applied to the tissue during maturation and can be segmented into the following zones: the superficial or tangential zone, the middle or transitional zone, the deep or radial zone, and the calcified zone. The ECM of the superficial zone mainly consists of thin collagen fibers that are lying parallel to the surface. The content of proteoglycans is low, which leads to a higher tissue permeability compared to the residual cartilage. The flattened chondrocytes in this area are accountable for appositional cartilage growth and are orientated parallel to the surface, closely associated to the collagen fibers. The middle zone features a lower cell density and the chondrocytes are configured roundly. The ECM in this layer is basically composed of type II collagen, which is organized into arcades, and proteoglycans in the highest concentration of all zones. The cells and collagen fibers of the deep zone and the calcified zone are arranged vertically to the subchondral bone. The cell density is lower than that in the other zones, while the proteoglycan content is relatively high. The presence of collagen X and hydroxyapatite in this area indicates chondrocyte maturation and calcification at the transition from cartilage to subchondral bone. The calcified zone functions as an interface between bone and cartilage to reduce the existing mechanical gradient [1], [3], [7].

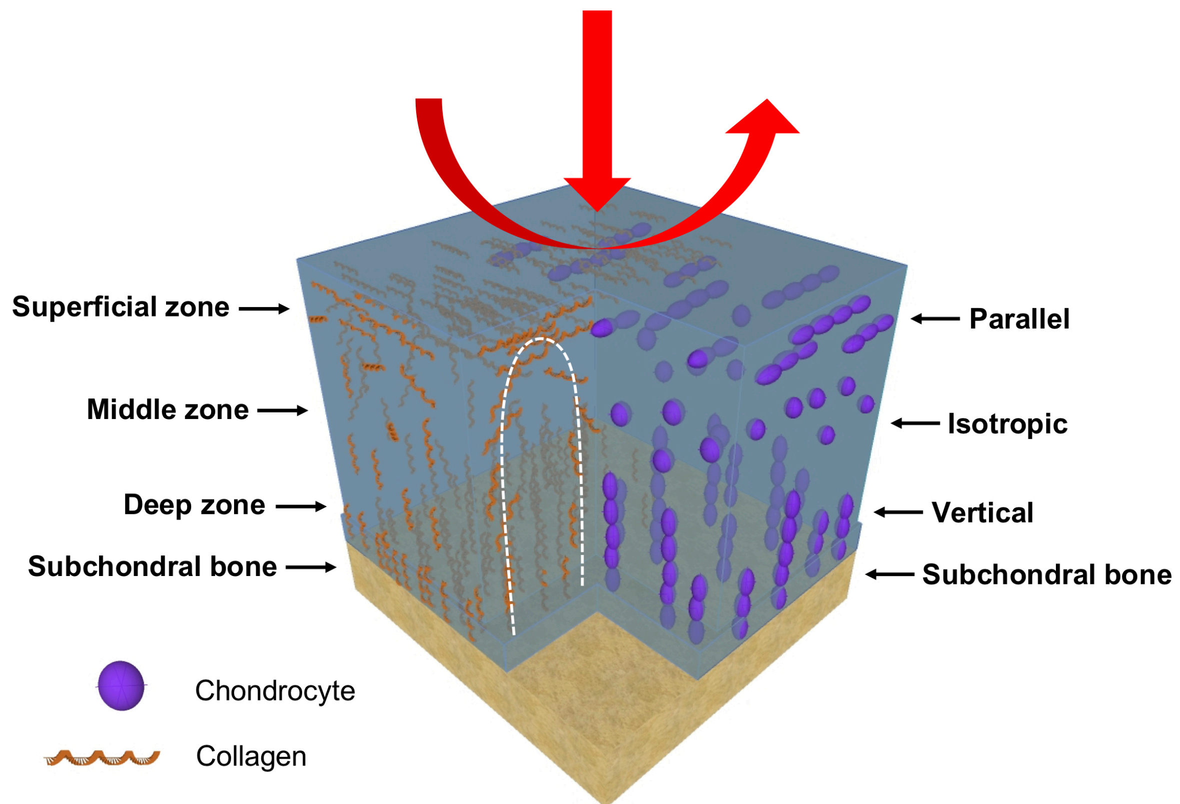


FIGURE 2 **Schematic representation of the multi-zonal structure of articular cartilage showing the collagen and cell orientation.** Collagen fibers are arranged in an arched pattern (dashed line). Application of compressive and shear forces to the articular surface (red arrows).

With its particular architecture of collagen fibrils articular cartilage is moreover capable of transmitting punctual loads equally to the subjacent bone area. This gives it a high resistance to sheer, tensile and compressive forces. The collagen fibrils, mainly collagen II with small additions of collagen IX and XI, are arranged arched like arcades and are anchored in the bone on both ends. Thus, mechanical pressure spreads over the whole collagen arch and in the sum of the fibrils this effect leads to an equal distribution of punctual stress to the subchondral bone.

Further articular cartilage simply acts as an effective shock absorber within the joint. This is based on its great content of proteoglycan (PG) aggregates, which are built off aggrecan and the electronegative glycosaminoglycans (GAG) hyaluronan, chondroitin sulfate and keratan sulfate. These aggregates are connected to the collagen fibrils. Because of the rejection of their negative loadings and the ability to incorporate water the PG aggregates would expand enormously but in the cartilage they are hold back by the tense collagen fibrils [FUGURE 1]. This allows barely a fifth of their possible dimension and results in the remarkable stability and pressure elasticity of the cartilage. The PGs can be compressed a bit indeed, but after decompression they

immediately expand to that limit given by the collagen fibrils, such as small elastic springs. The compression and decompression are accompanied by a corresponding flow of water into or out of the cartilage. But the low permeability of articular cartilage prevents the tissue from losing the fluid too quickly, which would reduce the shock absorbing effect [1]–[3], [6]. Apart from the biochemical skills cartilage also fulfills a paracrine function. Chondrocytes in articular cartilage influence each other through paracrine regulatory factors including TGFβ superfamily, Indian hedgehog homolog (IHH), parathyroid hormone related proteins (PTHrP) and Wnts. Thus articular chondrocytes are controlled by their environment not to undergo hypertrophy and to keep a stable chondrogenic state [8], [9]. Consequently disturbances of this paracrine signaling lead to abnormal articular cartilage development [10]–[12]. By comparison in growth plate cartilage IHH and PTHrP are secreted in the resting zone and the proliferation zone as well to inhibit hypertrophy and stimulate proliferation. But chondrocytes of the hypertrophic zone produce bone morphogenic proteins (BMPs) and Wnts to initiate hypertrophic differentiation of adjacent proliferating chondrocytes [8].

1.2 Acute cartilage injury

Injuries of the articular cartilage and the calcified chondral-subchondral bone region can have various possible causes and shapes.
According to the ICRS Hyaline Cartilage Lesion Classification System they can be classified into five grades [13]:

Grade 0: Normal cartilage
Grade 1: Superficial lesions. Soft indentation and/or superficial fissures and cracks
Grade 2: Lesions extending down to <50% of cartilage depth
Grade 3: Cartilage defects extending down to >50% of cartilage depth as well as down to calcified layer and down to but not through the subchondral bone. Blisters are included in this grade.
Grade 4: Osteochondral injuries, lesions extending just through the subchondral bone plate or deeper defects down into trabecular bone.

Articular injuries can be generated by a direct blunt trauma or an indirect or torsional loading. Blunt traumas could be hitting the joint with an optional object or falling on hard ground. Examples of indirect and torsional stress are leverage through a strike to a limb or an intense torsion of a loaded joint for example in sports like skiing or soccer. Generally speaking these are situations that frequently occur in sports and everyday live [7].
The mentioned injuries cause joint pain and limited range of motion and can evolve into osteoarthritis. Osteoarthritis is a degenerative joint disease that is characterized by a loss of articular cartilage and a degeneration of the subchondral bone as it lacks the protective cartilage layer.
Apart from the mentioned situations joint instability, insufficient joint or muscle innervation, a deficit in muscle strength or endurance, or a past joint surgery increase the risk of a degenerative joint disease as well. Even a successful anterior crucial ligament (ACL) reconstruction comes along with an increased risk for osteoarthritis [14]. As the disease can be traced back to a certain cause it's called secondary osteoarthritis. However osteoarthritis may also develop without an initial cause as a primary osteoarthritis for example because of the normal proceeding degeneration during the aging or long-term physical work [15].

To achieve the best possible results in treatment of osteoarthritis it is important to identify risk factors, which also include overweight, genetic predisposition and increased physical activity, and first symptoms in an early stage of the disease. Especially in early osteoarthritis, when clear clinical and radiographic signs may be limited, there might still be regenerative capability of the articular cartilage [16].

1.3 Repair capacity of articular damage

In general cartilage's potential to regenerate itself after damage has occurred is very limited. But if the injury restricts to the macromolecular framework such as disruption of collagen fibrils, the chondrocytes can react to the changed matrix composition with an increased production of new matrix molecules. Thus, chondrocytes are able to repair small defects to a certain degree. However, at the latest when the lesions reach macroscopically dimensions like chondral ruptures, flaps and tears the self-repair capacity of cartilage is exhausted. Although surrounding chondrocytes respond by proliferating and synthesizing extracellular matrix, they are not capable of filling the whole defect [7].

When it comes to the point, that subchondral bone is injured, the initial situation is another one. Blood from the bone marrow enters the injury site and fills the defect with a clot of fibrin and platelets. Platelets and bone matrix release growth factors and cytokines such as transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF), insulin-like growth factor I and II (IGF I & II) and bone morphogenic protein (BMP). These factors initiate vascular invasion and migration of undifferentiated mesenchymal stem cells into the defect and stimulate proliferation and synthetic activity of the chondrocytes. Soon after migration the stem cells start to differentiate chondrogenically and synthesize extracellular matrix consisting of collagen type I and II and a relatively high amount of proteoglycans. 6 to 8 weeks after the injury the newly formed tissue fills the whole defect. But in terms of structure and composition the repair tissue is more similar to fibrocartilage than to hyaline cartilage. Only in the rarest cases the repair tissue is able to form a sufficient joint surface. Larger osteochondral injuries – if untreated - show signs of degeneration including fragmentation, fibrillation, loss of chondrocytes and increasing collagen type I and collagen type X content. The remaining material appears as a fibrous tissue and even this mostly decomposes leaving the underlying bone bare and unprotected [7].

As one can see there is a high demand for adequate medical treatment of articular cartilage damages.

1.4 Current therapy methods for articular lesions

Currently different strategies are clinically used for treatment and repair of articular cartilage injuries:

1.4.1 Bone marrow stimulation

Based on the previously described repair mechanism of osteochondral defects methods have been developed to start that cascade of fibrin clot formation, stem cell migration and generation of fibrocartilage in articular lesions with conserved integrity of the subchondral bone. There are different variations among this technique such as subchondral drilling and microfracture. The microfracture as today's most common technique was developed by Steadman. Across the whole defect the subchondral bone plate gets perforated with a special awl. The microfractures are set at a distance of 3-4 mm to each other and down to a depth of 4 mm [17]. Unfortunately the fibrocartilage repair tissue ranks behind hyaline articular cartilage concerning biomechanical properties [7], [18] and paracrine function. But, particularly in young patients good clinical results with reduction of pain could be achieved [19]. This technique is adequate for treatment of small sized focal articular lesions.

1.4.2 Mosaicplasty (Osteochondral grafts)

This method was introduced in 1993 and since then frequently used for the therapy of chondral and osteochondral lesions [20]. Cylindric transplants composed of articular cartilage and subchondral bone are removed from healthy, low weight bearing articular areas and implanted into prepared holes in the defect zone. Good clinical results have been shown in several studies [20]–[22]. However, to receive the osteochondral grafts intact tissue has to be harmed which is a serious disadvantage of the therapy. Thus, the mosaicplasty is limited to small to medium sized focal defects. For larger defects allogeneic osteochondral allografts are available but limited in Germany due to regulatory specifications.

1.4.3 Autologous chondrocyte implantation

Since its first description in 1987 the autologous chondrocyte implantation (ACI) [23]–[25] has been continuously developed further and is used especially for the treatment of big chondral defects (>4 cm^2). The initial step of the method is to take a small biopsy of healthy chondral tissue out of a non-affected and low-weight-bearing part of the joint. The chondrocytes are divided from the ECM by enzymatic digestion and proliferated *in vitro*. Depending on the dimension of the defect it takes 2 to 3 weeks of cell culture to get enough chondrocytes for a sufficient therapy [26]. In the course of the latest modifications the chondrocytes are imbedded in a three-dimensional matrix and then implanted into the chondral defect [25], [27]. Different biomaterials have been tested for the matrix-associated autologous chondrocyte transplantation (MACT) including collagen scaffolds, hyaluronan-based biodegradable polymer scaffolds [28] and polymers of poly-actin and poly-galactin [29]. The matrix containing the seeded cells can easily be adapted to the size and form of the lesion. After implantation the chondrocytes start to form a hyaline-like repair tissue, which leads to significant improvement for the patients in the form of improved function and reduced pain [23]–[25]. Nonetheless there are considerable limitations for this technique. The leading problem is the restricted ability of differentiated chondrocytes to proliferate and therefor to generate an adequate number of cells. Additionally the risk of degenerative joint disease is increased by the removal of intact articular cartilage [25].

There is a certain range of possible treatments for articular cartilage defects. However, any of these practicable options has serious disadvantages. So, there is still a need for a therapy that offers a functional cartilage replacement with the biomechanical properties of physiological articular cartilage and without the necessity to harvest cells or crafts by damaging healthy cartilage. This claim might be solved by the use of mesenchymal stem cells.

1.5 Mesenchymal stem cells in cartilage repair

The introduction of mesenchymal stem cells (MSCs) with their ability to differentiate into cells of the chondrogenic lineage has started a new chapter in treatment of articular cartilage lesions. MSCs may have the potential to replace autologous chondrocytes in the therapy of cartilage defects and to provide even better results. Further on MSCs are available in huge numbers without the necessity of harming healthy cartilage tissue.

1.5.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a promising source for the regeneration of mesenchymal tissue such as articular cartilage. They can be harvested with relatively low effort from different tissues like bone marrow [30] or adipose tissue [31]. MSCs are very proliferative and can easily be grown *in vitro* without losing the ability to differentiate chondrogenically. As MSCs are multipotent cells they are able to differentiate into a range of different mesenchymal tissues. Depending on extracellular conditions such as the composition of the medium and added growth factors they can form bone, cartilage, tendon, muscle, fat, dermis and other connective tissues [30], [32]. The International Society for Cellular Therapy (ISCT) has proposed a set of standards to define MSCs. A cell can be classified as an MSC if it shows plastic adherent properties under normal culture conditions and has a fibroblast-like morphology. Cultured MSCs also express CD73, CD90 and CD105 on their surface, while lacking the expression of CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR surface markers [33].

1.5.1.1 Chondrogenic differentiation of MSCs

The high chondrogenic potential of MSCs has been proved in several different studies [34]–[37]. Furthermore there is a chance that they are able to recapitulate the embryonic lineage transitions originally involved in the formation of joint tissue [38].

Based on Johnstone’s *in vitro* chondrogenesis model for rabbit bone marrow derived MSCs [35] Yoo *et al.* developed a pellet culture system for human MSCs using a similar chondrogenic medium containing TGFβ, dexamethasone, ascorbate, pyruvate, proline

and ITS. Under this influence MSCs differentiate chondrogenically, which is distinguished by gene expression and synthesis of the chondrogenic markers collagen type II, collagen type IX, collagen type XI and aggrecan [36]. In this model TGFβ acts as the inducer of chondrogenesis. This is well established and confirmed by other authors [39], [40].

1.5.1.2 Hypertrophy in chondrogenesis of MSCs

Unfortunately, chondrogenically differentiating MSCs tend to undergo hypertrophy *in vitro* such as transient growth plate chondrocytes in long bones. Despite more than 20 years of research, this is one issue, that still makes them inappropriate for the clinical use in articular cartilage repair [35], [36], [40], [41]. The hypertrophic phenotype is characterized by an increased cell volume including a swollen appearance of the chondrocytes. The ECM passes through a remodeling process that is accompanied by an increasing content of collagen type X, the degradation of collagen type II and aggrecan by matrix metalloproteinases (MMP) 3 and 13 and the activity of alkaline phosphatase (ALP), which induces the calcification of the ECM [35], [36], [40]–[42]. Furthermore, after chondrogenically pre-culture and ectopic *in vivo* transplantation in mice human MSCs showed induction of hypertrophy followed by vascular invasion and terminal matrix calcification. For comparison implanted human articular chondrocytes kept a stable chondrogenic phenotype without signs of hypertrophy [43]. This indicates that there are differences in the developmental program of cultured chondrogenically differentiating MSCs and articular chondrocytes that have to be overcome to produce appropriate repair tissue. While the behavior of MSCs under chondrogenic conditions is similar to that of growth plate chondrocytes, which also undergo hypertrophy and develop into transient enchondral cartilage, the differentiation of articular chondrocytes autonomously stops before the hypertrophic stadium to form stable articular cartilage. The developmental analogies between chondrogenically differentiating MSCs and growth plate chondrocytes were further described by Mueller and Tuan by demonstrating that chondrogenic differentiation of MSCs is equal to that shown by chondrocytes during endochondral embryonic skeletal development [42].

In both cell groups genes acting as markers for chondrogenic differentiation and hypertrophy are regulated in a similar way. Additionally, their reaction to changes in medium composition is similar. Addition of thyroid hormone or bone morphogenic

protein-4 (BMP4) induces hypertrophy while TGFβ and dexamethasone inhibit hypertrophy in MSCs as well as growth plate chondrocytes [39], [42], [44]. Karl *et al.* proved, that the pro-hypertrophic effect of thyroid hormone is mediated by BMP4. Based on that they established an *in vitro* hypertrophy model for MSCs, which we adopt for our study. In this model hypertrophy in chondrogenically differentiating MSCs can be increased by withdrawal of TGFβ and dexamethasone and the addition of BMP4 [44]. We use this model to make the impact of the anti-hypertrophic treatment more distinct.

1.6 Endochondral ossification

Regarding the term of hypertrophy in chondrogenesis it is important to have a view at its physiological purpose in endochondral ossification. Endochondral ossification is the process behind the formation of vertebrate limb skeleton and length growth of long bones, which is characterized by the development of a chondral scaffold (growth cartilage) that is ultimately replaced by bone. Cartilage models (anlagen) are formed through condensation of mesenchymal cells that derive from three different sources: the neural ectoderm (cranial neural crest) for the craniofacial bones, the paraxial mesoderm for the axial skeleton and the lateral plate mesoderm for the skeleton of the limbs [45]. Mesenchymal cells in the center of the construct differentiate into highly proliferative pre-chondrocytes and express typical cartilage ECM molecules such as collagen II, IX, XI and aggrecan [46]. Cells in the outer zone of these ‘cartilage anlagen’ remain undifferentiated and form the perichondrium. Endochondral ossification in long bones takes place at two different ossification centers – the primary (diaphyseal) site and the secondary (epiphyseal) site in one or both end regions. Ossification starts in the diaphysis (primary site) with the formation of a bone collar that is built by perichondrium cells differentiating into osteoblasts. The epiphysis (secondary site) ossifies independently [46]. As an initial step a special subgroup of densely packed mesenchymal cells at the future joint site builds the avascular interzone which represents the first sign of joint formation. The interzone cells give rise to the permanent articular chondrocytes for both interlocking joint surfaces besides synovial lining, intra-articular ligaments and menisci. Meanwhile adjacent progenitor cells in the interrupted cartilaginous skeleton differentiate into chondrocytes that become organized in epiphysis and growth plate and undergo hypertrophy, vascular invasion and finally ossification [47]–[49].

Each cell population is characterized by distinct expression patterns. Whereas interzone cells express growth and differentiation factor 5 (GDF5), a ligand for the BMP receptor 1β (BMPR1β) [14], chondrocytes in epiphysis, diaphysis and growth plate are GDF5- negative and express matrillin 1 instead, which is not present in any stage of articular chondrocyte development [15]. Chondrocytes in growth plate cartilage are arranged in specific zones depending on their functional and developmental state. The initially small and round chondrocytes build the resting zone, which is furthest from the ossification front, with a source of resting chondrocytes that supplies the adjacent zone

of proliferation (proliferation zone). The chondrocytes become flattened and packed into parallel, longitudinal columns. The cells reduce their proliferation activity and pass through the transient stage of the 'pre-hypertrophic' chondrocyte (pre-hypertrophic zone). Finally, they completely undergo hypertrophy in the hypertrophic zone, which is characterized by massive increase of cell volume and a switch in protein production towards collagen type X, MMP3 & 13 and ALP that initiates the calcification of the ECM. Subsequently hypertrophic chondrocytes die leaving the calcified cartilage matrix surrounding them largely intact. Blood vessels, osteoclasts and osteoprogenitor cells invade the chondral model and use the remaining cartilage matrix as a scaffold for bone formation [46], [50]–[52]. Recent studies were able to show that terminal differentiated chondrocytes in endochondral ossification are capable to survive, redifferentiate and contribute as osteoblasts to the then required bone formation [53]. During adolescence endochondral ossification still occurs in the growth plate, which is responsible for the longitudinal growth of the bones and clearly demonstrates the division of chondrocytes into different zones according to their maturation stage.
Endochondral ossification is also necessary for secondary fracture healing as the callus, a cartilaginous template that is initially built in the fracture zone, is replaced by bone [54]. But apart from endochondral ossification there is another possible way of bone formation, the intramembranous ossification, which is responsible for the development of flat bones (e.g. the cranium). In contrast to endochondral ossification intramembranous ossification is not in need of a cartilage scaffold. After condensation the mesenchymal cells directly differentiate into osteoprogenitor cells. Maturated osteoblasts then produce and calcify the bone matrix [55].

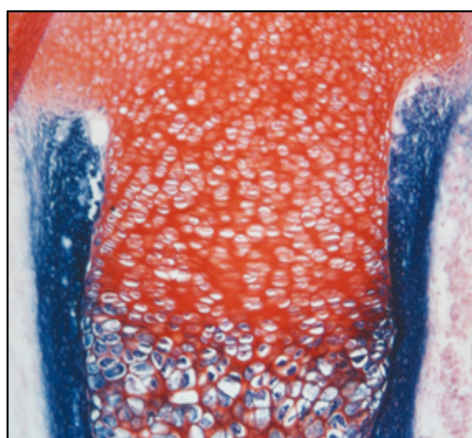


FIGURE 3 **Zonal structure of the growth plate during endochondral bone development.** Mouse tibial growth plate. ALP staining (blue), neutral red as counterstaining (Source: www.histologyworld.com).

1.7 RAR-Pathway

Retinoid signaling plays a key role in chondrocytes maturation and bone formation during limb skeletogenesis by regulating genes that influence cell growth, differentiation, survival and death.

While retinoids induce hypertrophy and mineralization in the late stage of growth plate development [56], they are also capable of attenuating chondrogenesis in the early stage [57], [58]. Shimono *et al.* were able to inhibit heterotropic ossifications by orally administered retinoic acid receptor agonist treatment in mice. This effect was most likely traced back to the inhibition of the initial chondrogenic differentiation of MSCs as an inevitable early step of heterotropic ossification [59]. This correlates to the findings of Ballock *et al.* who showed that retinoic acid treatment of epiphyseal chondrocytes in monolayer culture results in a decreased expression of collagen II and aggrecan and an increased expression of the matrix metalloproteinases stromelysin-1 (MMP3) and collagenase 3 (MMP13) [60].

However, as endogenous retinoids are mainly distributed through the blood vessel system they are supposed to physiologically impact growth plate ossification mainly in the late stage, when blood vessels are sprouted into the growth plate cartilage [59], [61], [62]. In early skeletogenesis suppression of RAR signaling by unliganded RAR seems to be a crucial requirement for chondrogenesis and expression of pro-chondrogenic genes and growth factors including Sox genes and BMPs [63]–[66].

There are two different subfamilies of intracellular retinoid receptors which are both located in the nucleus: the retinoic acid receptors, RAR α, β and γ, on one side and the retinoic X receptors, RXR α, β and γ on the other. There are two more RAR isoforms, RAR δ1 and δ2, however they cannot be found in human, but in amphibian stem cells [67], [68].

The RXRs respond to 9-cis-retinoic acid, a physiological isomer of all-trans-retinoic acid (tRA), and also act as heterodimeric partner for several nuclear receptors such as vitamin D, thyroid hormone and retinoic acid receptors [69].

In the nucleus RAR and RXR form heterodimers and bind to specific retinoic acid response elements (RARE) located in the promoter area of several specific target genes. They operate as ligand-dependent transcription factors modulating target gene expression. In addition non-activated, non-liganded receptor complexes are involved in chromatin condensation, which inhibits gene transcription [70].

RARγ seems to be the most important retinoid receptor for limb development. Mutant mice that are deficient in RARα or RARβ expression show normal phenotypes whereas the growth plates of RARγ deficient mice are defective and lack in aggrecan expression and content [71]. According to this finding RARs show distinct differences in gene expression during enchondral ossification. Both, different timing and distinct spatial distribution is found. Whereas RARα expression remains broad and diffuse and RARβ is restricted to the perichondrium, RARγ is highly expressed in hypertrophic cells and expression is selectively up-regulated just before the chondrocytes undergo hypertrophy [58].

The RARs interact with different co-regulators. Depending on the binding ligand bonds to co-activators (CoA) or co-repressors (CoR) are reinforced or weakened [72]. Further on CoAs are necessary for correct binding of agonists and downstream signaling. RAR-CoR constructs however handicap the binding of agonists and therefore decrease or even inhibit downstream RAR signaling.

In avascular cartilage zones RARs exert ligand-less receptor function. Non-liganded RARs built complexes with CoR, which represses target gene transcription by probably promoting chromatin condensation. Under retinoid free conditions RARs, especially RARγ, are beneficial for proteoglycan expression in chondrocytes [70], [73].

While inactive vitamin A is stored in the liver, released retinol is bound to plasma retinoid binding proteins (RBPs) forming a hydrophobic complex on their way to target cells. In many tissues retinol is transported through the cell membrane by the specific RBP receptor STRAT6 (“stimulated by retinoic acid” RBP receptor/Vit. A transporter) and intracellular transferred into active all-trans retinoic acid by retinol dehydrogenases (RDHs) and retinaldehyde dehydrogenases (RALDH) [66], [74]–[76].

Intracellular retinoids in cytoplasm are associated to special binding proteins that are involved in cell uptake of retinoids, protect them from non-specific interactions, control intracellular retinoid distribution and transport them between cellular compartments. The cellular retinoic acid binding protein II (CRABPII) transfers retinoic acid to the nucleus and delivers it to RAR by “channeling” via direct protein-protein contact without dissociation of the ligand into the aqueous phase. Thus CRABPII promotes formation of the RAR-RA complex and stimulates the RA-induced transcriptional activity of RAR [77]–[79].

The binding of the physiological agonist tRA to RAR effects the release of CoR and the binding of CoA, which leads to chromatin modification and subsequent activation

of gene expression for example of the transcriptional factor MafB and proteins involved in the Wnt/β-Catenin pathway. This is followed by expression of the matrix metalloproteinases MMP3 and MMP13 as a sign of hypertrophy in chondrocytes [72], [80]. Jimenez *et al.* furthermore demonstrated that the RA induced expression of MMP13 is mediated by *runt-related transcription factor 2* (Runx2) as targeted deletion of the Runx2 gene in mice nearly completely inhibited MMP13 gene expression [81].
In our model we analyze the impact of the treatment with a special type of retinoid antagonist, BMS204,493 (further named BMS), which works as a pan-RAR-inverse agonist (binds to all three RARs). As an inverse agonist (IA) BMS has to be distinguished from neutral antagonists that are only capable of competitive replacement of agonists. BMS causes a more active process and increases CoR interactions compared to the unliganded receptor state [72], [82], [83].

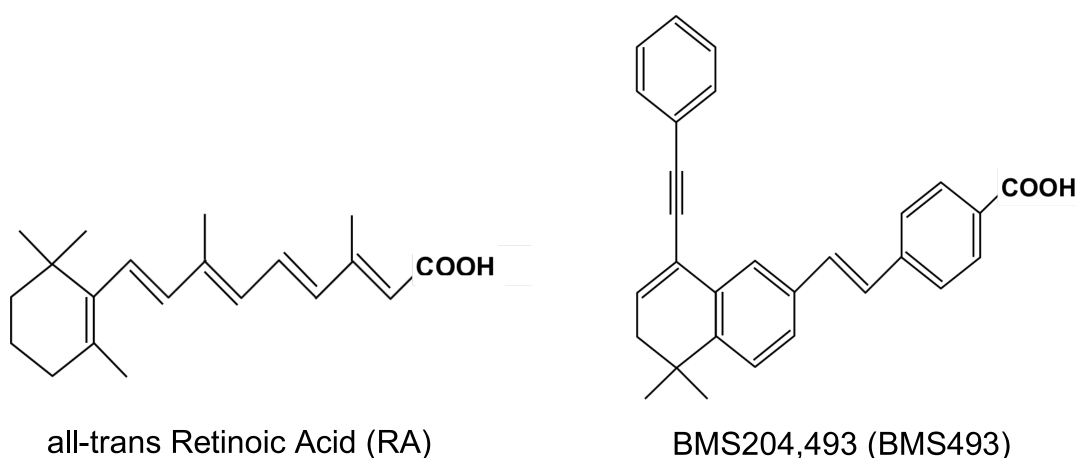


FIGURE 4 **Structures of the RAR agonist all-trans retinoic acid and the pan-RAR inverse agonist BMS493**

1.8 Wnt/β-Catenin signaling

In chondrocytes one important pathway to interact with the RAR pathway is the Wnt/β-Catenin signaling pathway. Through activation of this pathway gene expression of aggrecan and collagen II is inhibited, and expression of metalloproteinases is activated, which leads to matrix degradation in cartilage.

Retinoid signaling stimulates Wnt/β-Catenin signaling as Wnt, particular Wnt 2b and 5a, and Wnt receptors and co-receptors (Frizzeleds and lipoprotein receptor related protein (LRP) 5/6) are target genes of the RAR pathway.

When Wnt proteins bind to cell surface complexes of these receptors and co-receptors, phosphorylation of β-Catenin by glycogen synthase kinase 3 (GSK3) decreases. Non-phosphorylated β-Catenin doesn't undergo degeneration through the proteasome pathway and accumulates in the cytoplasm.

After translocation to the nucleus it interacts with lymphoid enhancer factor/T cell factor (Lef/Tcf) transcription factors and activates expression of direct and indirect target genes. Runx2 [84] and subsequently MMPs and collagen type X expression is up-regulated [85], whilst SOX9 (*SRY related HMG box 9*) [86] and therefore aggrecan and collagen type II expression is decreased [87].

Yasuhara and co-workers showed that both retinoic acid (RA) and Wnt3a treatment inhibits gene expression of Aggrecan and up-regulates expression of MMP13 and MMP3 in chondrocytes by stimulating Wnt/ β-Catenin signaling. Co-treatment with RA and Wnt3a enhance these effects [88]. However, RA treatment decreases Wnt/ β-Catenin signaling in other mesenchymal cells. Thus, responses to retinoid and Wnt/ β-Catenin signaling are supposed to change during chondrogenic cell differentiation as a sign of distinct temporal signaling mechanisms.

Some results indicate that under retinoid free conditions RARs inhibit Wnt/ β-Catenin signaling in chondrocytes and thus act as pro-chondrogenic receptors [88]. Figures 5 and 6shows possible cross-regulations between the two pathways.

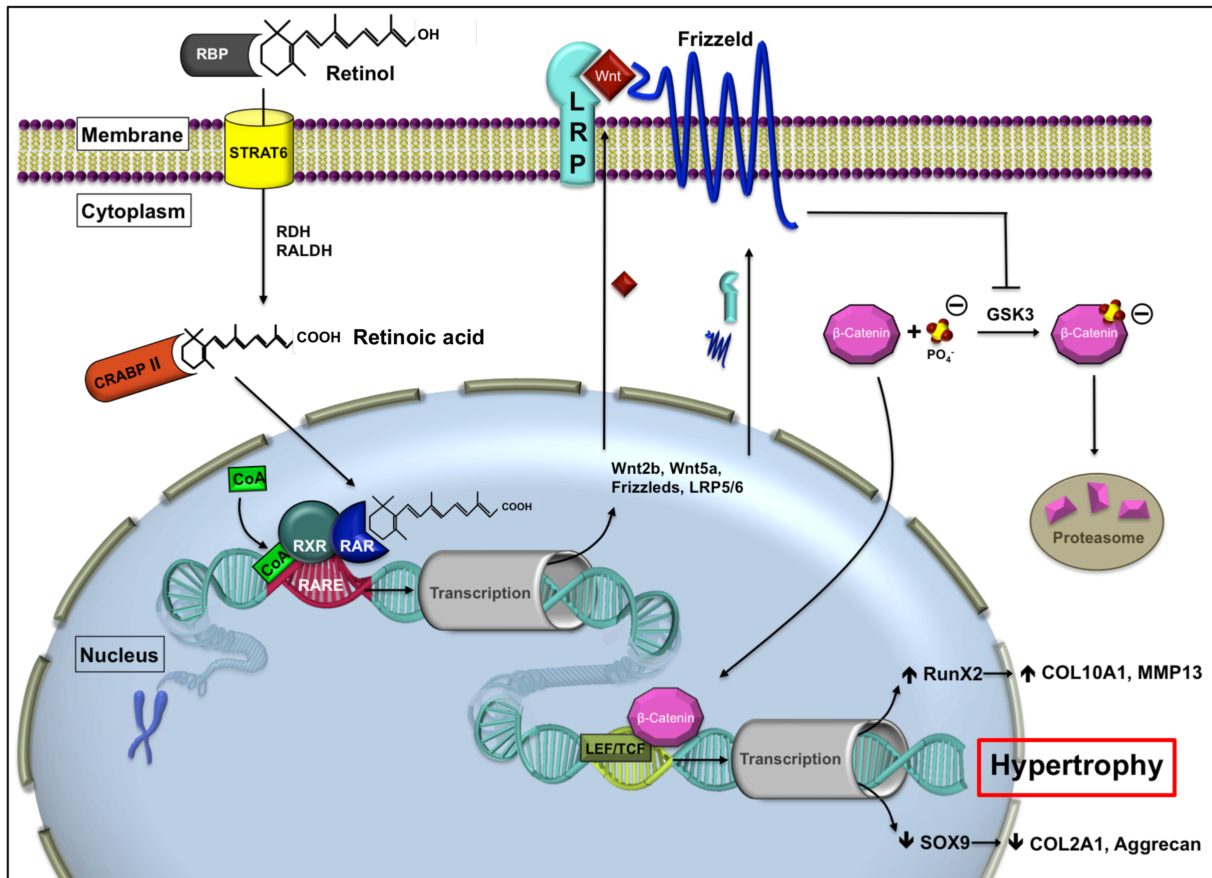
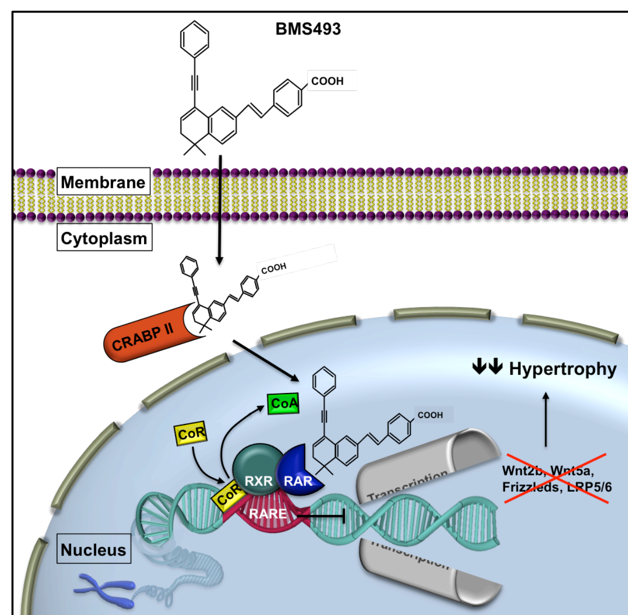


FIGURE 5 **Possible connections of Wnt/ß-catenin and retinoid signaling pathways**.
The retinoic acid is translocated into the nucleus by CRABP II. Binding of retinoic acid (RA) to the RA receptor would activate gene expression of Wnt proteins, receptors, and co-receptors which leads to an increased Wnt/ß-Catenin signaling followed by hypertrophic conversion.
CoA co-activator **CRABP II** cellular retinoic acid binding protein **GSK3** glycogen synthase kinase 3 **LEF/TCF** lymphoid enhancer factor/T cell factor **LRP** lipoprotein receptor related protein **MMP13** matrix metalloproteinase 13 **RALDH** Retinaldehyde dehydrogenase **RAR** retinoic acid receptor **RARE** retinoic acid response element **RBP** retinoid binding protein **RDH** retinol dehydrogenase **RunX2** runt-related transcription factor 2 **RXR** retinoic X receptor **SOX9** SRY related HMG box 9 **STRAT6** “stimulated by retinoic acid” RBP receptor/Vit. A transporter

FIGURE 6 **Schematic demonstration of the inhibition of the RAR pathway by BMS493**
BMS493 is translocated into the nucleus by CRABP II and binds to the RAR/RXR complex. Binding of the inverse agonist leads to a destabilization of co-activator bounds and supports co-repressor recruitment. The receptor complex subsequently inhibits target gene expression at the promoter area RARE. The reduced expression of Wnts and Wnt receptors and co-receptors decreases hypertrophic differentiation. **CoA** co-activator **CoR** co-repressor **CRABP II** cellular retinoic acid binding protein **GSK3** glycogen synthase kinase 3 **LEF/TCF** lymphoid enhancer binding factor/T cell factor **LRP** lipoprotein receptor related protein **RAR** retinoic acid receptor **RARE** retinoic acid response element **RXR** retinoic X receptor



1.9 TGFβ superfamily

The transforming growth factor β superfamily is a big group of signaling molecules encoded by 33 genes. Its name is derived from the first member of the family to by isolated. Apart from TGFβ the group includes bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and inhibins. Members of the family, which are expressed in both vertebrates and invertebrates, can be found in nearly any tissue and operate from the very first steps of development throughout lifetime of living beings. They play elementary roles in the regulation of various biological processes such as cell growth and differentiation, pattern formation and regulation of the immune system [89], [90].

1.9.1 TGFβ signaling

An important requirement for the initiation of chondrogenesis and the prosecution of chondrogenic differentiation is the activity of TGFβ signaling and further downstream pathways. The pro-chondrogenic effect of TGFβ *in vitro* and *in vivo* has been demonstrated in several studies. TGFβ signaling promotes chondrogenic differentiation yet inhibits chondrocyte hypertrophy and terminal differentiation [91]–[97]. Accordingly TGFβ expression is activated in the early stage of chondrogenesis but not in hypertrophic chondrocytes [98], [99].

There are two subtypes of TGFβ receptors, TGFβR1 and TGFβR2, which form a heterodimeric serine/threonine kinase receptor complex. The signaling starts with the binding of ligands to the receptor complex. TGFβR2 phosphorylates TGFβR1, which again phosphorylates and activates the associated *Sma and Mad related proteins* (SMADs), SMAD2 and SMAD3. After phosphorylation and translocation to the nucleus SMAD2/3 cooperate in a complex with SMAD4 acting as a transcriptional factor, that up-regulates the expression of the target gene SOX9. SOX9 is essential for the initiation of chondrogenesis and furthermore for the maintenance of chondrogenic differentiation. Simultaneously TGFβ signaling in general is down-regulated during hypertrophic conversion in chondrocytes [100], [101].

SOX9 forms a complex with SMAD2, SMAD3 and SMAD4 that promotes target gene expression. During chondrogenic differentiation SOX5 and SOX6 are co-expressed

with SOX9 and necessary as co-factors for an adequate expression of collagen type II, aggrecan and other cartilage specific genes [102]–[105].
The TGFβ pathway is modulated on different levels. SMAD6 and SMAD7 have an inhibiting effect on the activity of SMAD2/3 and consequently reduce TGFβ signaling. The membrane anchored molecules betaglycan and endoglin both exert influence on the TGFβ pathway by binding TGFβ subtypes but with converse outcome. While betaglycan enhances TGFβ signaling endoglin seems to reduce TGFβ signaling [106]. Additionally TGFβ signaling is dependent on the activation of latent TGFβ as all three subtypes, TGFβ1, TGFβ2 and TGFβ3, are synthesized in an inactive form [107].

1.9.2 BMP signaling

Further members of the TGFβ superfamily that fulfill a crucial function in limb development are bone morphogenic proteins (BMPs). The subfamily of BMP related proteins contains over 20 multi-functional cytokines, however especially BMP2, 4, 6 and 7 are known to affect endochondral ossification [108]–[111]. The exact role of BMPs is far from defined but various studies suggest them to be an important part of the regulation mechanism of cartilage formation and development. BMP signaling is supposed to promote aggregation of mesenchymal cells into pre-chondrogenic condensations [112] and to stimulate chondrogenic differentiation and expression of chondrogenic markers such as collagen type II and aggrecan [113]–[116]. Although BMPs might have a protective effect in articular cartilage, they also seem to be involved in chondrocyte hypertrophy and matrix degradation. Several *in vitro* and *in vivo* studies showed that BMPs effected an increased hypertrophic conversion in chondrocytes including increased expression of hypertrophic markers like collagen type X and ALP and that inhibition of BMP signaling prevents chondrocytes hypertrophy [109]–[111], [116]–[120]. According to this Karl and Mueller developed a BMP4 based hypertrophy model for pellet culture of MSCs [44], which we use in our study as well.

BMP signaling is mediated by a dimeric transmembrane receptor complex built of type I and type II serine/threonine kinase receptors. Analogously to TGFβ signaling ligand binding starts a cascade of phosphorylation within the receptor complex, which concludes with the activation of intracellular signaling molecules (SMAD1, 5 and 8).

Activated SMAD1/5/8, form a complex with SMAD4 to regulate target gene expression in the nucleus [121], [122].

The ways of regulating the BMP pathway are diverse. Similarly, to TGFβ signaling the inhibitory SMAD6 and 7 decrease phosphorylation of SMAD1/5/8. Extracellular BMP-specific antagonists such as noggin, chordin, follistatin and gremlin can influence the level of ligand availability by complexing with BMPs and preventing them from interacting with their receptor [123]–[125]. Furthermore, the pseudoreceptor BAMBI (BMP and activins membrane-bound inhibitor) disturbs the interactions between type I und type II receptor of the TGFβ superfamily as it is a transmembrane protein structural similar to the type I receptor but without enzymatic activity. During embryonic development BAMBI is co-expressed with BMP4 and therefore functions as a negative feedback regulator for BMP signaling [126].

BMP signaling leads to an up-regulation of Runx2 expression [127], [128]. Runx2, also called core binding factor subunit α1 (CBFA1), belongs to the *runt*-domain gene family, whose members are characterized by the DNA-binding domain *runt* [129]. It acts as a stimulator of hypertrophy in differentiating chondrocytes as various hypertrophic markers including collagen type X [130] and MMP13 [131] were identified as Runx2 targets. Furthermore Runx2 cooperates with the BMP associated transcription factors SMAD1/5/8 to induce collagen type X gene expression [130]. Runx2 expression increases corresponding to chondrocyte differentiation. The gene expression level of Runx2 is low in resting and proliferating chondrocytes and highest in terminal maturated hypertrophic chondrocytes [132]–[134].

2 Aim of the study

Mesenchymal stem cells (MSCs) seem to be the right candidate cells for the treatment of articular cartilage lesions. They can easily be obtained in appropriate quantity and have the potential to differentiate chondrogenically. But regrettably, current models for *in vitro* chondrogenesis of MSCs induce a hypertrophic differentiation that is similar to the developmental program of growth plate chondrocytes during enchondral ossification rather than to pheno- and genotypically stable articular chondrocytes. So, as we are looking for a stable and functional replacement tissue for cartilage defects and hypertrophy would result in apoptosis and ossification the concern for tissue engineering applications for cartilage repair using MSCs rises. Before MSCs can be used adequately in cartilage repair, the *in vitro* chondrogenesis protocols have to be improved to a point, where hypertrophy can be attenuated completely and permanent chondrogenic differentiation can be reliably induced.

In order to find a way to inhibit hypertrophy in this study we concentrate on the RAR-pathway. Retinoid signaling plays a key role in chondrocytes maturation during limb skeletogenesis. As retinoids induce hypertrophy and mineralization and attenuate chondrogenic differentiation it could have a positive effect on chondrogenesis of MSCs to inhibit RAR signaling. To achieve a RAR inhibition we use a special antagonist called BMS493, which acts as a pan-RAR inverse agonist. In our study we analyze if BMS treatment has an impact on hypertrophy of chondrogenically differentiated human MSCs (hMSCs) *in vitro*. And since the effect of retinoic acid to growth plate chondrogenesis depends on the state of differentiation we investigate if BMS application at different time points leads to different outcomes.

3 Materials and methods

3.1 Materials

3.1.1 Recombinant Proteins

Chemical	Application	Concentration	Company
BMP4	BMP ligand	25 ng/ml	R&D systems
TGFβ1	TGFβ ligand	10 ng/ml	R&D systems
BMS204,493	Pan-RAR inverse agonist	2 µM	Sigma

TABLE 1 Recombinant Proteins

3.1.2 Primers

Primers were synthesized by eurofins. Solutions of 100 µM (100 pmol/µl) were prepared and stored at -80°C. The following primers were used.

Gene	Sequence (forward)	Sequence (reverse)	Concentration
VPS29	AGCTGGCAAACTGTTGCAC	GACGGTGGTGGTGACTGAG	200 nM
PSMB4	GCTTAGCACTGGCTGCTTCT	GGACATGCTTGGTGTAGCCT	200 nM
REEP5	AGGTCAGCCACTGGGTATCA	CCTCTCTCCTCTGCAACCTG	200 nM
MMP13	GACTGGTAATGGCATCAAGGGA	CACCGGCAAAAGCCACTTTA	200 nM
COL1A1	ACGTCCTGGTGAAGTTGGTC	ACCAGGGAAGCCTCTCTCTC	200 nM
COL2A1	GGGCAATAGCAGGTTCACGTA	TGTTTCGTGCAGCCATCCT	200 nM
COL10A1	CCCTCTTGTTAGTGCCAACC	AGATTCCCAGTCCTTGGGTCA	200 nM

TABLE 2 List of primers. Genes are abbreviated according to the NCBI gene database

3.1.3 Antibodies

Primary antibodies		
Antibody	**Dilution**	**Company**
Collagen type II (mouse)	1:100	Calbiochem
Collagen type X (mouse)	1:20	Quartett Immunodiagnostica
Secondary antibodies		
Goat-ant-rabbit (HRP-conjugated)	1:1000	Pierce
Goat-anti-mouse (biotinylated)	1:100	Dianova
TABLE 3 List of antibodies		

3.1.4 Kits

Name	Application	Company
Alkaline Phosphatase Kit	ALP staining	Sigma Aldrich
Brilliant SYBR Green QPCR Master Mix	qPCR	Agilent Technologies
DC Protein Assay	Protein concentration	Biorad
Quant-it Pico Green dsDNA-Kit	DNA concentration	Invitrogen
RNeasy Plus Universal Kit	RNA clean up, DNA digestion	Qiagen
Transcriptor First Strand cDNA Synthesis	cDNA synthesis	Roche
Vectastain Elite ABC HRP Kit	Immunohistochemical Collagen staining	Vector Laboratories
Pierce DAB Substrate Kit	Immunohistochemical Collagen staining	Thermo Fisher Scientific
TABLE 4 List of kits		

3.1.5 Buffers and solutions

Tris 0,2 M (pH 7,0)

24,2 g Tris base

add H_2O to 1l

Washing Buffer

780 ml Tris 0,2 M (pH 7,0)

2,4 g NaCl

60 ml Triton X100

1% FCS

1% goat serum

add H_2O to 250 ml

Blocking Buffer

120 ml Tris 0,2 M (ph 7,0)

2,4 g NaCl

60 ml Triton X100

1% FCS

1% goat serum

add H2O to 250 ml

McIlvain Buffer (pH 3,6)

6,8 mM Citric acid

6,4 mM Na2HPO4

PBS

137 mM NyCl

2,7 mM KCl

10 mM Na_2HPO_4 x 2 H_2O

2 mM KH_2PO

Phosphate-Buffer (0.2 M)

29,7 g Na_2HPO_4

4,6 g NaH_2PO_4

add H_2O to 1l

ALP Buffer

1,5 M Tris (pH 9,0)

1 mM Mg Cl

1mM ZnCl

3.1.6 Cells

Human mesenchymal stem cells (MSCs) derived from the iliac crest of male patients aged under 60 (n=3) were used for the experiments. Cell harvest was approved by the ethical committee of Regensburg University Medical Center end performed after written information and consent of the patient. Cells were made anonymous after harvest for further investigation.

3.1.7 Cell culture media

Proliferation medium:

Dulbecco's modified Eagle's medium (DMEM) low glucose (Invitrogen) with 10% fetal calf serum (PAN Biotech GmbH) and 1% penicillin/streptomycin (Invitrogen).

Differentiation medium:

Chondrogenic medium: DMEM high glucose (Invitrogen), 1% ITS+3 (Sigma Aldrich), 100 nM dexamethasone (Sigma Adrich), 40 µg/ml L-proline (Sigma Aldrich), 50 µg/ml ascorbate-2-phosphat (Sigma Aldrich), 10 ng/ml TGFβ1 (R&D Systems).

Hypertrophic medium: DMEM high glucose, 1 % ITS+3, 50 µg/ml ascorbate-2-phosphate, 40 µg/ml L-proline, 25 ng/ml BMP4 (R&D Systems)

Test group medium additionally contained 2 µM BMS204,493 (Sigma Aldrich).

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Isolation of MSCs

Human MSCs were isolated from bone marrow aspirates of male patients' iliac crest undergoing surgery that required autologous bone grafting with approval of the ethics committee of the University of Regensburg and written consent. MSCs were isolated by Ficoll gradient centrifugation. Therefore, bone marrow medium mixture was carefully layered onto a Ficoll (Biochrom) cushion in a 50 ml Falcon tube. The Falcon tube was then centrifuged for 35 minutes at 1680 U/min. The cell pellet, in which MSCs are located, was collected from the 1,077 g/ml density interface and mixed with fresh proliferation medium. After a further centrifugation step (10 min, 1000 U/min), the pellet was resuspended in fresh medium, the cell number was determined, and the cells were plated at a density of 2 million cells per 75 cm^2

3.2.1.2 Expansion of MSCs

MSCs were cultured as monolayer in 15 ml proliferation medium in 75 cm^2 tissue culture flasks. Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. Medium changes were performed every three to four days and at 80 % confluence cells were trypsinized and frozen for later use in liquid nitrogen.

3.2.1.3 Differentiation of MSCs

The chondrogenic differentiation of the MSCs was initiated according to the *in vitro* chondrogenesis model established by Johnstone et al [35]. MSCs of passage two were used for the experiments.

After unfreezing MSCs were seeded in 75 cm^2 tissue flasks at a number of approximately 500.000 cells per flask and expanded in proliferation medium until 80% confluence was reached. Cells were washed with sterile PBS and 3 ml trypsin (PAN Biotech GmbH) was added to remove the cells from the surface under gently shaking. The trypsin was inactivated with fresh proliferation medium and the cell suspension was then centrifuged for 5 minutes at 1000 U/min. The cell clot was resuspended in

DMEM high glucose medium and cell concentration was determined. Cells were seeded in V-bottomed 96-well polypropylene plates at a number of 200.000 per well and pellets were formed by centrifugation at 250 g for 5 minutes. Cells were chondrogenically differentiated in chondrogenic medium (as indicated in 3.1.7) for 14 days. During this phase one group of aggregates additionally was treated with BMS (2 µM). After these 14 days of chondrogenic pre-differentiation, pellets were split into seven groups and cultivated for another 14 day under different medium conditions (as described in 3.2.1.4).

MSC aggregates were kept at 37 °C in a humidified atmosphere containing 5 % CO_2. Medium was changed three times per week. MSC pellets were isolated at different time points and specified histologically, histochemically and immunohistochemically. Furthermore, ALP activity, GAG content, gene expression and protein expression were analyzed.

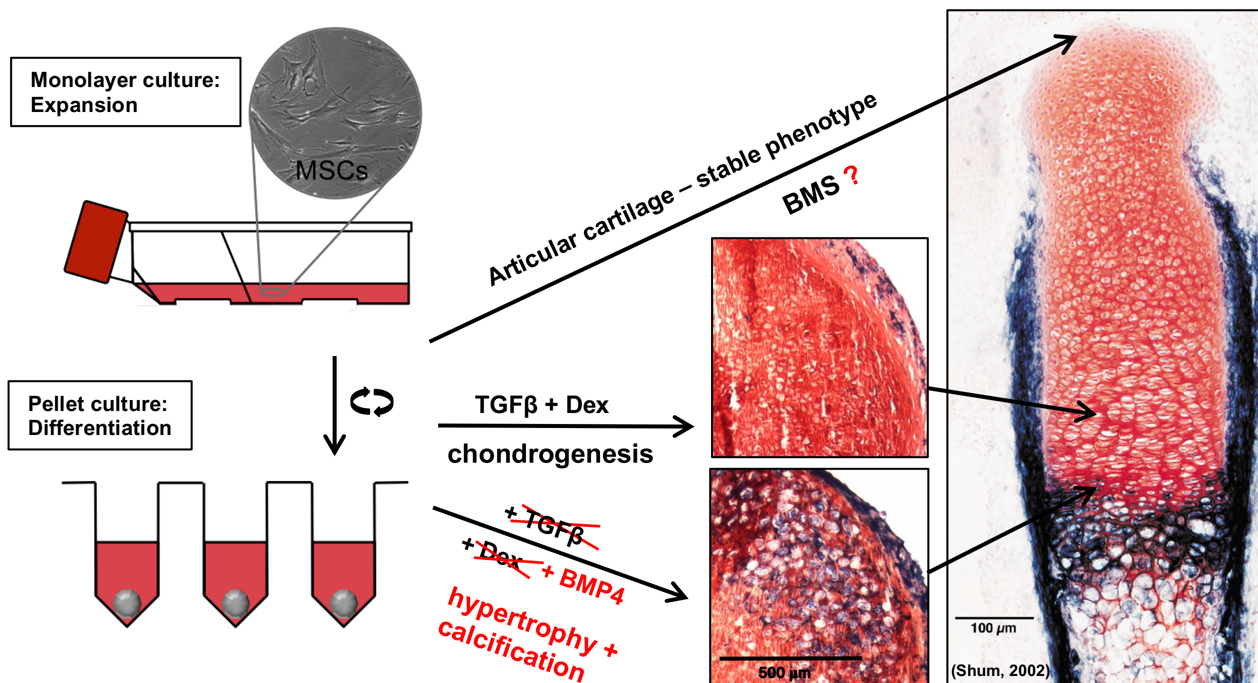


FIGURE 7 **Sequence of the cell culture** Human mesenchymal stem cells are isolated from bone marrow aspirates of the iliac crest of different donors. The cells are expanded in monolayer culture and transferred into 96-well plates at passage 2. Pellets are formed by centrifugation and incubated in differentiation medium for 4 weeks. Chondrogenesis can be induced with a well established chondrogenic medium containing TGFβ and dexamethasone. Furthermore, hypertrophy can be stimulated by withdrawal of TGFβ and dexamethasone and addition of BMP4.

3.2.1.4 Modulation of hypertrophy

MSC pellets were formed as described and differentiated in chondrogenic medium for 14 days treating one part of cells additionally with BMS (as indicated in 3.2.1.3). On day 14 aggregates were divided into seven different groups with different proliferation media forming pairs of one respectively two test groups and one associated control group, which differ in the addition of BMS to the test groups' medium:

(1) control group: chondrogenic medium; (2) test group: chondrogenic medium with 2 μM BMS493;

(3) control group: chondrogenic medium without TGFβ1; (4) test group: chondrogenic medium without TGFβ1 but with 2 μM BMS493;

Note: The established chondrogenic medium contains TGFβ1 because of its chondrogenic potential. Through this test constellation we want to investigate, if BMS, which is also supposed to have a chondrogenic effect, is able to compensate the lack of TGFβ1 and generate a chondrogenic phenotype.

(5) control group: hypertrophic medium (as indicated in 3.1.7); (6) test group A: hypertrophic medium with 2 μM BMS493; (7) test group B: addition of 2 μM BMS493 to the chondrogenic medium only during predifferentiation and switching to hypertrophic medium on day 14;

Note: Through these two hypertrophic test groups the effect of BMS treatment during different phases of chondrogenesis can be investigated.

Cells for gene expression analysis were isolated on day 0, day 1, day 14 and day 28. For GAG assay aggregates were harvested on day 1, day 14, and day 28. Aggregates for histological analysis and medium supernatant for ALP activity analysis were isolated on day 14 and day 28.

Figure 8 shows the line-up of the different test and control groups and their different medium conditions.

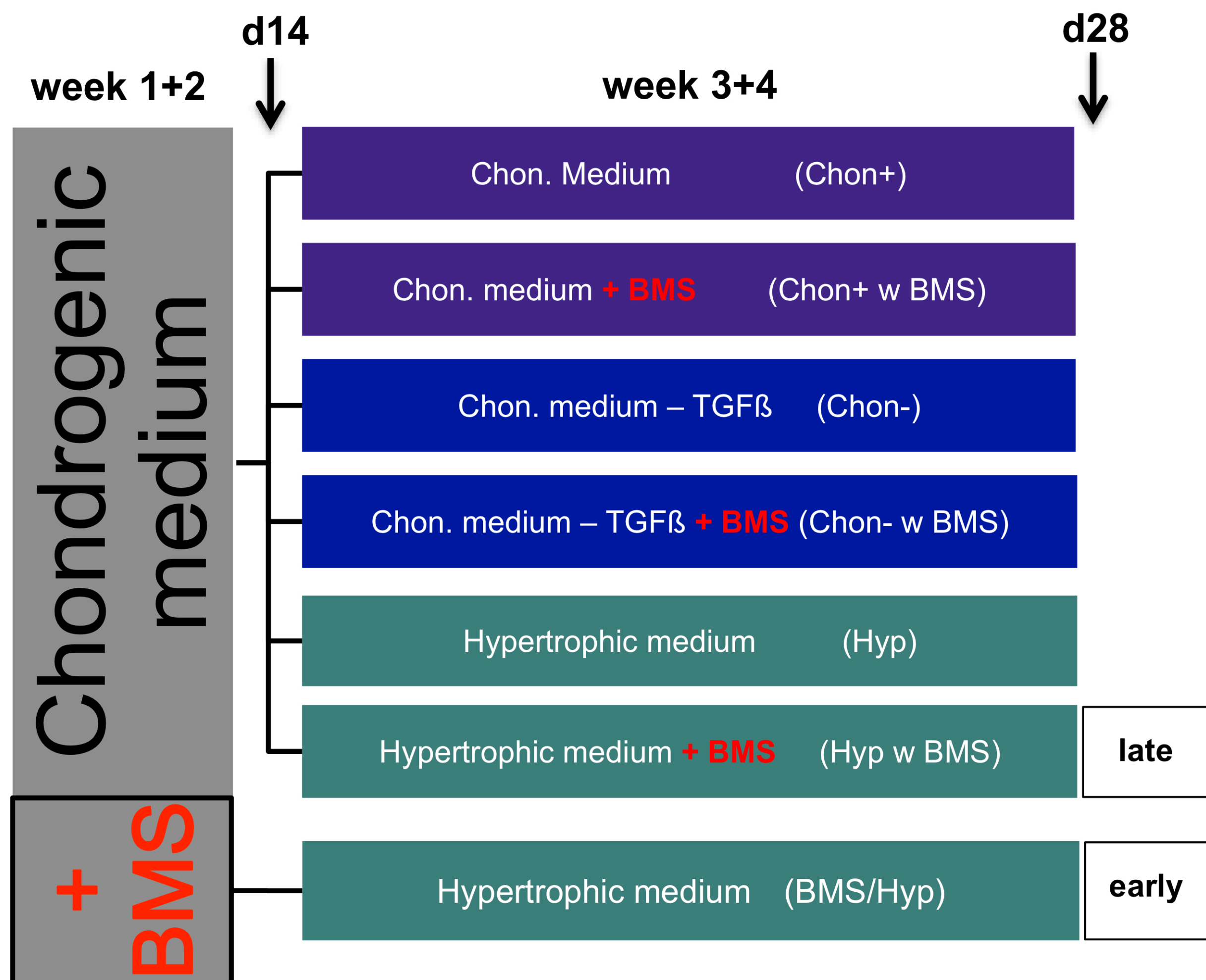


FIGURE 8 **Classification of Aggregates into different test and associated control groups with different composition of proliferation medium.** Late and early BMS group additionally marked.

3.2.2 Histology, Histochemistry and Immunohistochemistry

3.2.2.1 Fixation of MSC pellets and preparation of frozen sections

MSC aggregates were isolated on day 14 and day 28 and fixed in 4% paraformaldehyde for one hour. Then the pellets were rinsed with 0,1 M phosphate buffer and incubated for an hour each in sucrose solutions of increasing concentration (10%, 20%, 30% sucrose solution in 0,1 M phosphate buffer). Afterwards MSC aggregates were embedded in TissueTec (Sakura) and frozen in liquid nitrogen. 10 µm thick cryo sections were prepared with the Microm HM 500 OM Cryotom (Microm, Berlin, Deutschland).

3.2.2.2 DMMB staining

1,9-Dimethylmethylenblue (DMMB) stains sulfated glucosaminoglycans (GAGs) that form a main part of the cartilage extracellular matrix and are synthesized by chondrocytes during chondrogenesis. Frozen sections were rehydrated in distillated water for 5 minutes and incubated in 0,1% DMMB solution (Sigma Aldrich) for 10 minutes. Afterwards sections were washed twice with distillated water and dehydrated with alcohol of increasing concentration (1x 90% propanol, 2x 96% propanol, 2x 100% propanol, 5 minutes each) and Xylol (2x 100% Xylol, 5 minutes each) as final step. Cover slips were attached with DePex solution (Serva Electrophoresis GmbH). Sections were analyzed under the microscope at 4x and 10x magnification.

3.2.2.3 Alkaline phosphatase (ALP) staining

For ALP staining an alkaline phosphatase kit was used according to manufacturer's instructions. Neutral red (3-Amino-7-dimethylamino-2-methylphenazinhydrochlorid) was used as counterstaining. Cover slips were attached with 70% sorbitum solution (Caelo). Sections were analyzed under the microscope.

3.2.2.4 Immunohistochemistry

Immunohistochemistry was used for collagen type II and X staining. Therefore, Sections were rehydrated in washing buffer for approximately 10 minutes. Then endogen peptidases were inhibited with 3% H2O2/ 10% Methanol in PBS for 30 minutes. Sections were rinsed three times with washing buffer. Before blocking pepsin digestion for antigen preparation was carried out for 15 minutes at room temperature. For collagen type X staining an additional hyaluronidase digestion for one hour was performed before pepsin digestion. Then sections were incubated in blocking buffer for one hour at room temperature, followed by incubation in a solution of the particular primary antibody in blocking buffer overnight at room temperature. Immunolabeling was detected with a biotinylated secondary antibody, horse reddish peroxidase (HRP) conjugated avadin (Vector Laboratories) and enhanced diaminobenzidine (DAB) as substrate (Sigma).

3.2.3 Microscopy

To investigate the stained sections following microscope was used: Nikon ECLIPSE TE 2000-U

3.2.4 Gene expression analysis

3.2.4.1 RNA isolation and cDNA synthesis

For gene expression analysis aggregates of every culture condition were isolated on day 28. Additionally, aggregates of group 1 and group 7 were harvested on day 1 and day 14. Furthermore, the cells of two tissue flasks on day 0 had been isolated, before pellets were formed. 8 to 10 pellets per condition and per time point as well as the cells of day 0 were pooled, incubated in 0,9 ml Qiazol (Qiagen) and homogenized using the Precellys24 homogenizer (bertin instruments). The aqueous phase was transferred into a fresh tube and RNA was isolated using the RNeasy Plus Universal Kit (Qiagen). In brief, initially 100 µl gDNA-Eliminator solution and then 180 µl chloroform were added and the tube was shaken for 15 seconds after each step. Then the solution was incubated at room temperature for 15 minutes and centrifuged for 15 minutes at 12000 g and 4°C. Supernatant (approximately 0,6 ml) was again isolated and mixed with at least an equal amount of 70% Ethanol. The solution was then filtrated in special tubes by centrifugation at 8000 g and eluted in 30 µl RNase free water. RNA concentration was measured using the 2100 Bioanalyzer (Agilent Technologies).
1 µg RNA was reverse transcribed in cDNA using the Transcriptor First Strand cDNA Sythesis kit (Roche) according to manufacturer's instructions.

3.2.4.2 Real time polymerase chain reaction (PCR)

Gene expression was analyzed by semiquantitative real time PCR. Therefore, a real time PCR Detection System (CFX96, Biorad) was used in combination with Brilliant SYBR Green QPCR Master Mix (Agilent Technologies). The optimal primer concentration for each gene was identified through previous tests and is listed in table 2. cDNA was diluted to a concentration of 1 ng/µl. For PCR a final volume of 20 µl was used containing 10 µl Brilliant SYBR Green QPCR mix, 0,2 µl forward primer, 0,2 µl reverse primer, 4,6 µl RNase free water and 5 µl of the cDNA sample. PCR was

performed under following conditions. Initially DNA was denatured at 95°C for 15 minutes followed by 40 cycles of alternating denaturation (10 second, 95°C) and primer annealing and extension (30 seconds, 60°C). Melting temperature of the PCR product was analyzed by continuous measurement of fluorescent intensity during slow heating from 55°C to 95°C. Gene expression was normalized to following reference genes: Vacuolar Protein Sorting 29 (VPS29), Proteasome Subunit Beta Type 4 (PSMB4) and Receptor Expression Enhancing Protein 5 (REEP5).

3.2.5 Biochemical analysis

3.2.5.1 GAG analysis

Sulfated glycosaminoglycan (GAG) content relativized to DNA content was used as a quantitative marker for chondrogenic differentiation. For GAG analysis 4 to 5 aggregates were harvested on day 1, day 14 and day 28 and digested in 200 µl papain digestion solution (150 µg/ml Papain (Sigma) in PBS, 6 mM L-Cystein (Merck), 6mM EDTA (Sigma), pH 6.0) at 60°C over night. GAG content was determined with the DMMB method and chondroitin sulfate A from bovine trachea (Sigma Aldrich) was used as a standard. DNA content was measured with the Quant-it Pico Green dsDNA-Kit (Invitrogen) according to manufacturer's instructions.

3.2.5.2 Alkaline phosphatase activity

ALP activity was determined densitometrically by measuring the change in the absorbance at 405 nm through the transformation of p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate [108]. Therefore, medium supernatant was isolated on day 14 and day 28 and centrifuged for 5 minutes at maximum speed. 75 µl of supernatant was transferred to a 96-well flat bottom plate and 75 µl substrate solution (4mg/ml p-nitrophenol phosphate (Sigma Aldrich) in ALP buffer) was added. Continuous absorbance at 405 nm was measures spectrophotometrically in a Genius plate reader (Tecan) at room temperature. The change in A_{405} over time (dA/min) was calculated in the linear range of the reaction.

3.2.6 Statistical analysis

The data from real time PCR analysis, ALP activation analysis and GAG content analysis were expressed as mean values ± standard deviation (SD). Each experiment was performed with cells of marrow preparations of three different donors. The one-way Anova Test in SPSS statistic software (IBM) in combination with the Tukey Post-Hoc-Test was used for statistical analysis. A level of $p<0.05$ was considered significant.

All methods had been previously described in parts [44].

4 Results

4.1 Differentiation of MSC aggregates under chondrogenic and hypertrophic conditions

To start with the basis for our actual experiments we investigated MSC aggregates that had been treated with chondrogenic differentiation medium on different time points histologically, biochemically and on gene expression level and compared them to MSC aggregates under hypertrophic medium conditions.

4.1.1 Over time development of MSC aggregates under chondrogenic conditions

Besides the pellets obtained at the end of cell culture on day 28 we also isolated MSC aggregates on day 14 and cells on day 1 to track the course of the differentiation under established chondrogenic medium conditions. Therefore, histological analyses and real time PCR were carried out.
Histologically the aggregates showed a distinct progression of chondrogenesis over a period of two weeks. While the cell density of pellets on day 14 (FIGURE 9 A, B, C, D) is high because of a low content of ECM the volume of the aggregates has been multiplied on day 28 (FIGURE 9 E, F, G, H) and there was a large area of ECM around each cell. The content of GAGs was equal at both time points (FIGURE 9 B, F) however collagen type II staining was much stronger and more homogenous on day 28 (FIGURE 9 G) compared to aggregates on day 14 (FIGURE 9 C), which indicates the production of a cartilaginous ECM. However, MSC pellets on day 28 also show first signs of hypertrophic conversion. ALP staining was detected in the outer zone of the aggregates (FIGURE 9 E) and immunohistochemical analysis results in an appreciable presence of collagen type X in the whole pellet on day 28 (FIGURE 9 H), whereas pellets on day 14 were free of these hypertrophic markers (FIGURE 9 A, D).
The histological results were confirmed by gene expression analysis of pellets on day 1, 14 and 28. The collagen type II gene expression was statistically significantly upregulated from day 14 to day 28 whereas collagen type I gene expression was on a constant level during the whole cell culture. Analogous to immunohistochemical staining expression of the hypertrophic marker collagen type X was statistically significantly increased from day 14 to day 28 however there were no statistically

significant differences in the gene expression of the second hypertrophic marker MMP13 between any time points.

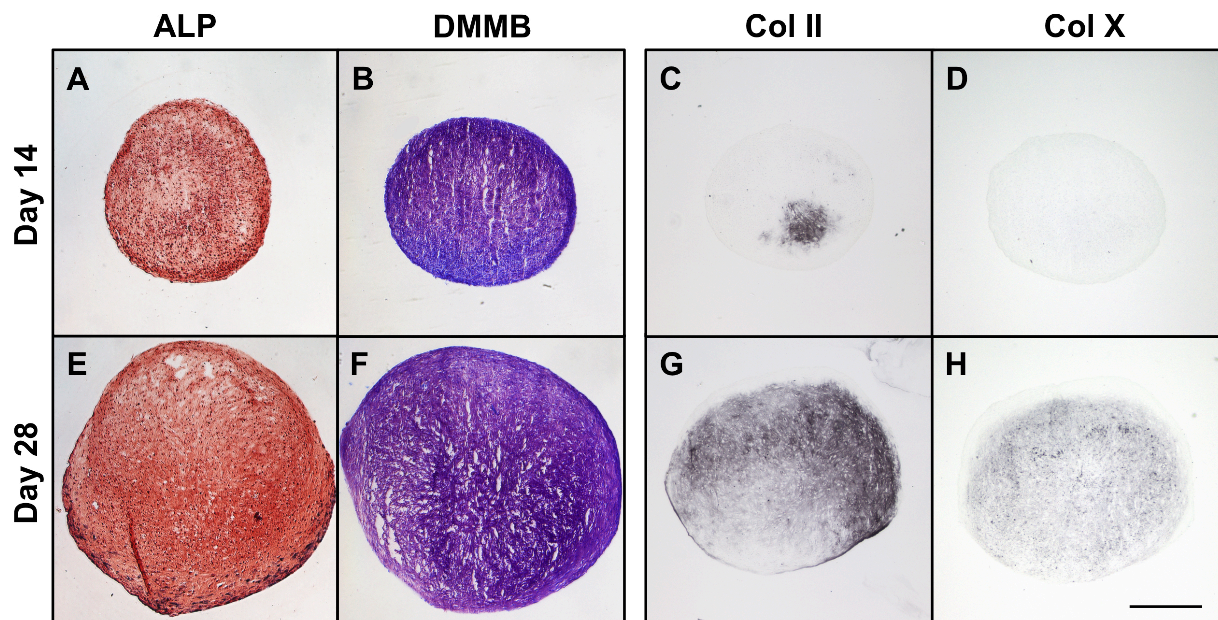


FIGURE 9 **Histological Appearance of MSC aggregates on day 14 (A, B, C, D) and day 28 (E, F, G, H) of pellet culture under chondrogenic conditions. A, E** alkaline phosphatase (ALP) staining (blue) with neutral red as counterstaining. **B, F** DMMB staining. **C, G** immunohistochemical collagen type II staining. **D, H** immunohistochemical collagen type X staining. Scale bar = 500 µm.

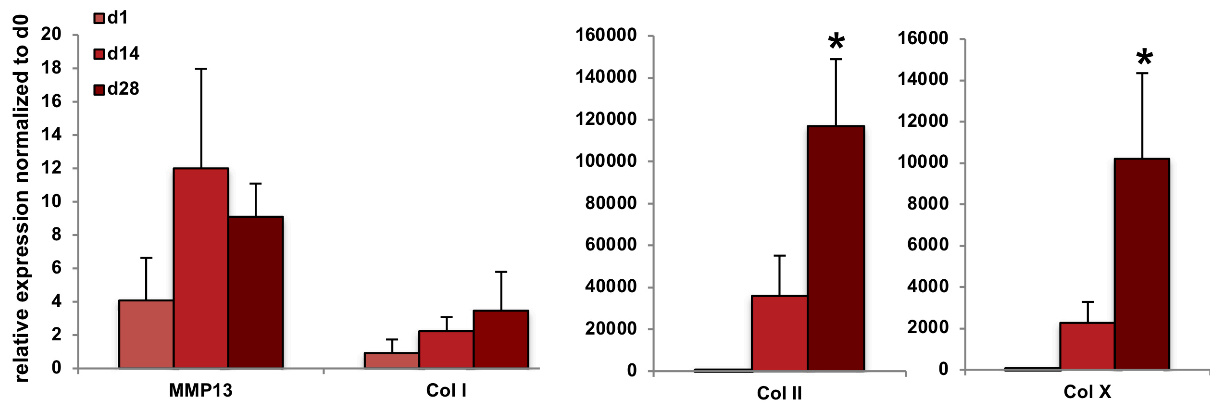


FIGURE 10 **Gene expression analysis of MMP13, collagen type I, collagen type II and collagen type X relativized to PSMB4, REEP5 and VPS29 and normalized to day 0 of MSC aggregates on day 1, 14 and 28 of cell culture under chondrogenic conditions analyzed by real time PCR.** MMP13 and collagen type I gene expression show no significant differences between any time points. Collagen type II and X expression is statistically significantly upregulated from day 14 to day 28. n=3 different donors.

4.1.2 Effect of hypertrophy enhancing medium conditions on MSC aggregates

4.1.2.1 Histological analysis

Under chondrogenic conditions aggregates on day 28 showed a homogenous morphology consisting of small, round cells that are surrounded by a uniform ECM very similar to hyaline cartilage structure. In comparison MSCs under hypertrophy enhancing conditions especially in the outer zone of the aggregates showed a distinct increase of cell volume and large intracellular lacunae, which are typical characteristics of hypertrophic cartilage. DMMB staining and immunohistochemical staining for collagen type II are strong in chondrogenic (Figure 11 B+C) as well as hypertrophic MSC aggregates (Figure 11 F+G). However, these two stainings are at least well suited to demonstrate the differences in morphology. Collagen type X staining, which is a marker for hypertrophy, showed distinct differences between chondrogenic and hypertrophic groups. While chondrogenic aggregates (Figure 11 D) featured only a slight staining, collage type X was clearly increased under hypertrophic conditions (Figure 11 H) particularly in the periphery of the aggregates. ALP staining (blue) was strong in hypertrophic areas and at the edge of the hypertrophic aggregates (Figure 11 E) but limited to a thin frame around the aggregates under chondrogenic conditions (Figure 11 A). Unfortunately, the counterstaining with neutral red was very strong so that it may cover the blue ALP staining in a certain way, but at a closer look the blue staining in and around the hypertrophic cells was clearly visible (Figure 12 B).

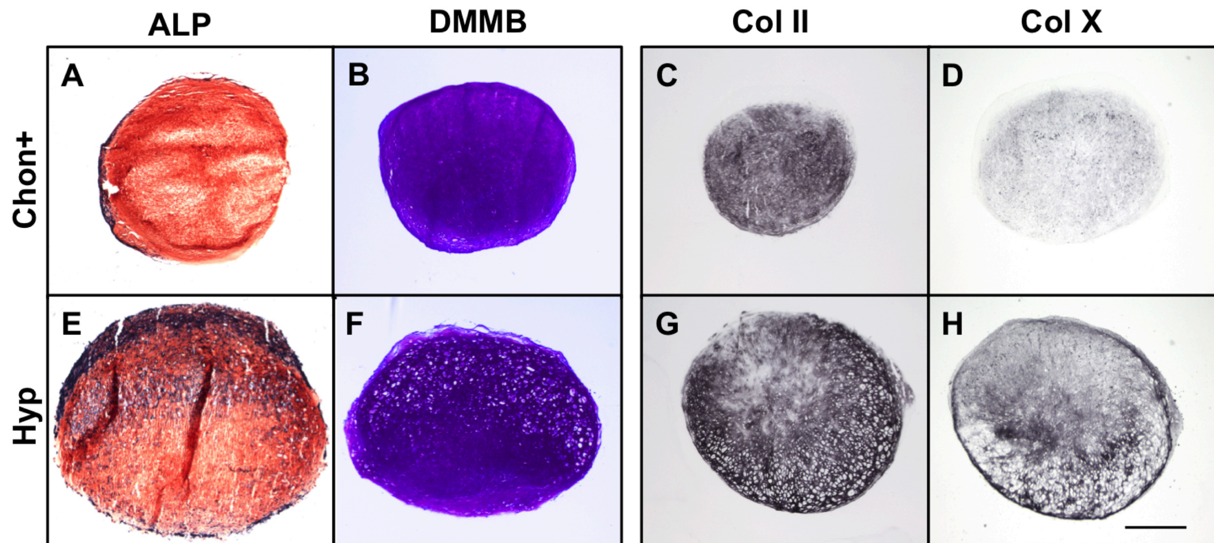


FIGURE 11 **Histological Appearance of MSC aggregates on culture day 28 under chondrogenic (A, B, C, D) and hypertrophy enhancing conditions (E, F, G, H). A, E** alkaline phosphatase (ALP) staining (blue) with neutral red as counterstaining. **B, F** DMMB staining. **C, G** immunohistochemical collagen type II staining. **D, H** immunohistochemical collagen type X staining. Scale bar = 500 µm.

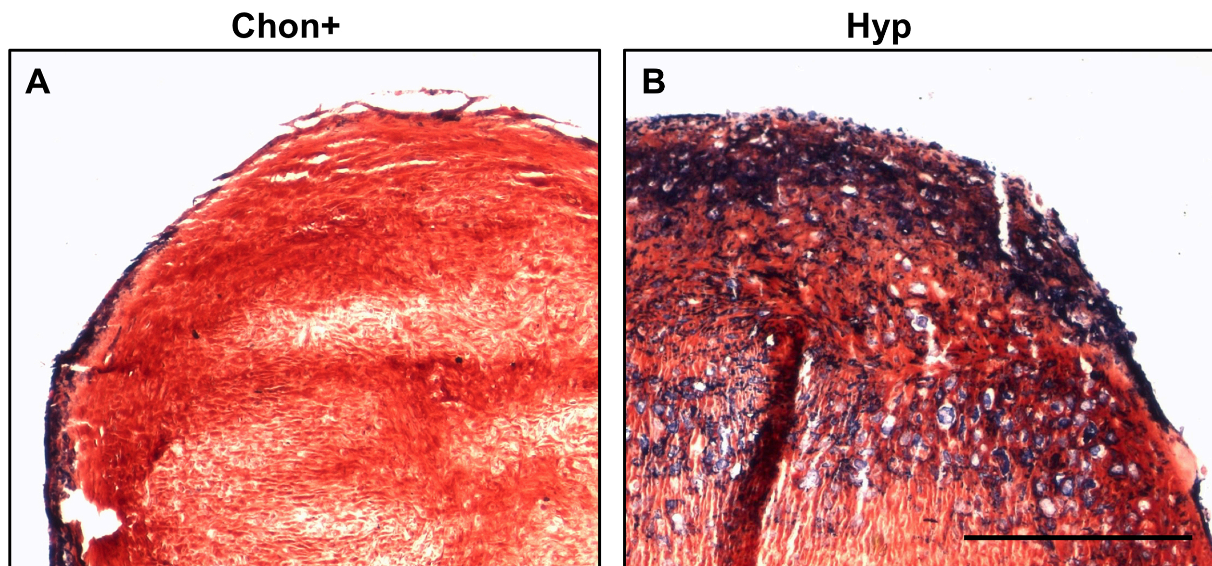


FIGURE 12 **Alkaline Phosphatase (ALP) staining with neutral red counterstaining of MSC aggregates on culture day 28 under chondrogenic (A) and hypertrophy enhancing conditions (B)** Under hypertrophy enhancing conditions ALP staining is strong on the edge of the aggregate. Neutral red counterstaining may cover some of the blue ALP staining but the blue color in and around the hypertrophic cells is clearly visible. Scale bar = 500 µm.

4.1.2.2 Gene expression analysis

To compare chondrogenic and hypertrophic aggregates on gene level we investigated the gene expression of the chondrogenic marker collagen type II, the hypertrophic markers collagen type X and MMP13 and the osteogenic marker collagen type I by

real time PCR analysis. Similar to the histological results collagen type II expression was equal in chondrogenic and hypertrophic conditions (FIGURE 13 B). As well as in collagen type I expression (FIGURE 13 A) no significant differences could be detected. Gene expression of COL1A1 was on a very low level throughout every time point and medium condition, which actually is an expected result and conformable to several other studies [135]–[137] that showed that collagen type I expression is high in undifferentiated mesenchymal cells and rapidly decreases in a very early stage of chondrogenic differentiation, which means immediately after transfer of the cells into the differentiation culture. Collagen type I gene expression stays down-regulated during chondrogenesis until final hypertrophic maturation stages.

In contrast to immunohistochemical staining for collagen type X no statistically significant differences in collagen type X expression could be found between chondrogenic and hypertrophic aggregates (FIGURE 13 B). Merely gene expression analysis of the hypertrophic marker MMP13, which is involved in degradation of cartilage ECM proteins, revealed statistically significant differences between chondrogenic and hypertrophic groups (FIGURE 13 A). Aggregates that were kept under hypertrophy enhancing conditions expressed a higher level of MMP13 compared to chondrogenic MSC pellets.

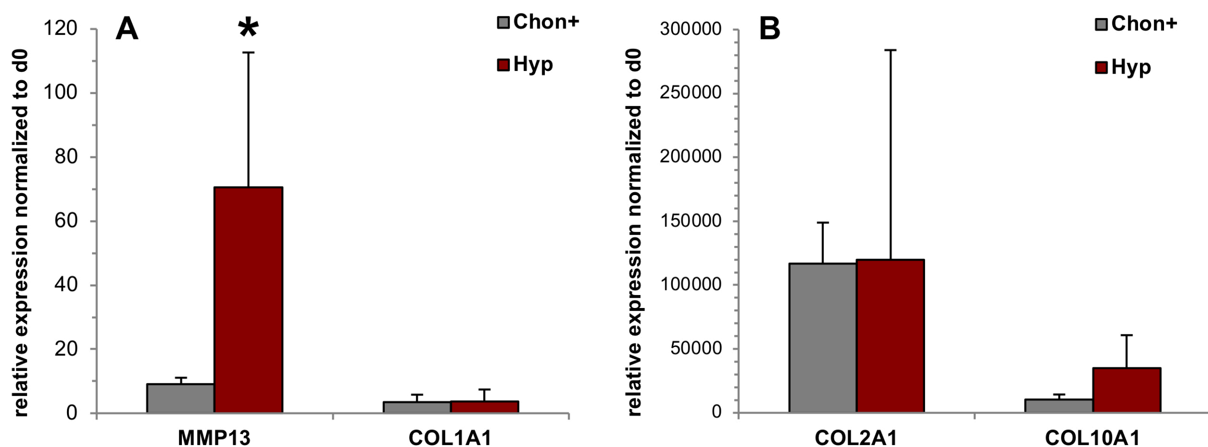


FIGURE 13 **Gene expression analysis of MMP13, COL1A1, COL2A1 and COL10A1 relativized to PSMB4, REEP5 and VPS29 and normalized to day 0 of chondrogenic and hypertrophic MSC aggregates analyzed by real time PCR.** Collagen type I (A), collagen type II (B) and collagen type X (B) expression show no statistically significant differences between chondrogenic and hypertrophic groups. MMP13 expression (A) is statistically significantly upregulated under hypertrophy enhancing conditions compared to chondrogenic conditions. n=3 different donors.

4.2 Attenuation of hypertrophy upon treatment with BMS493

As the RAR pathway plays a key role in growth plate hypertrophy, we aimed to investigate in this experiment weather inhibition of RAR signaling by treatment of chondrogenically differentiating MSCs with the RAR inverse agonist BMS493 may have influence on chondrogenesis and especially on hypertrophy in MSCs.

4.2.1 Histological analysis

Under chondrogenic conditions with continuous application of TGFβ BMS treatment had small, but histologically visible effect on chondrocytes phenotype compared to chondrogenic control aggregates. Both groups showed a hyaline cartilage-like morphology with little indication of hypertrophy. Aggregates with BMS treatment were free of ALP positive cells (FIGURE 14 B) while chondrogenic control aggregates exhibited ALP staining in their outer zone (FIGURE 14 A). DMMB staining was a bit stronger in the BMS group (FIGURE 14 F). In immunohistochemistry chondrogenic aggregates with BMS treatment showed a higher content of collagen type II (FIGURE 14 K), which is an evidence for the pro-chondrogenic effect of BMS. Collagen type X staining in the chondrogenic BMS group was nearly equal to control aggregates, however more concentrated at the edge of BMS aggregates (FIGURE 14 O). Under TGFβ free conditions after chondrogenic pre-differentiation long term chondrogenic differentiation just occurred to a smaller extent. The weak DMMB staining and neutral red counterstaining in ALP aggregates indicate a small content of sulfated GAGs. Apart from that, the ECM contains nearly no collagen type II (FIGURE 14 L) compared to the chondrogenic groups. The application of BMS had no positive impact on chondrogenesis, actually it seems to inhibit chondrogenesis in a certain way based on the weaker DMMB and collagen type II staining (FIGURE 14 H, M). Thus, these results disagree with the hypothesis, that BMS treatment allows a proper chondrogenesis in absence of TGFβ.

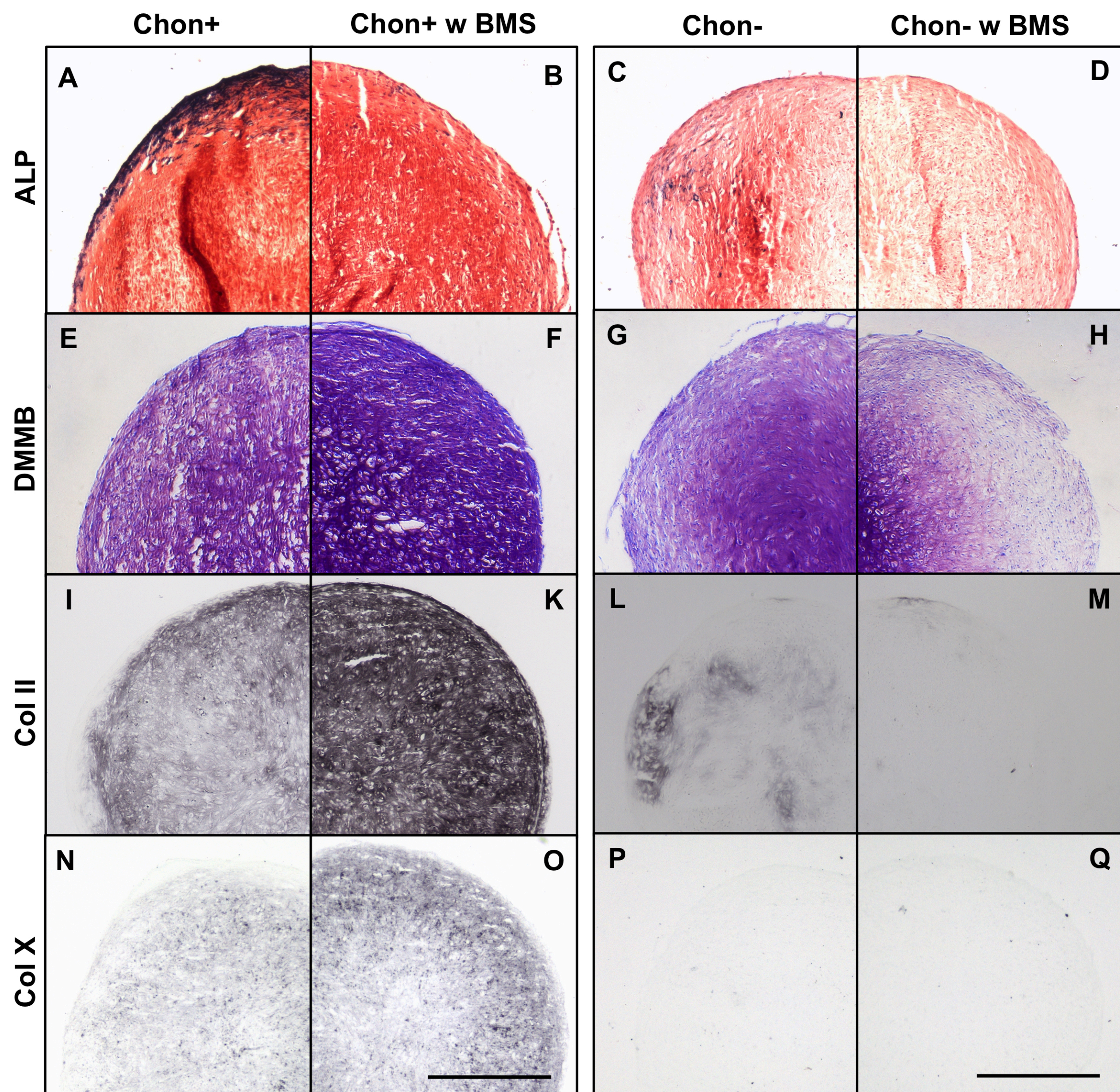


FIGURE 14 **Histological appearance of MSC aggregates on day 28 after BMS treatment under chondrogenic conditions respectively in absence of TGFβ1. A, E, I, N** Chondrogenic control group (Chon+). **B, F, K, O** Test group with BMS treatment from day 14 (Chon+ w BMS). DMMB staining (**E, F**) and immunohistochemical collagen type **II** staining (**I, K**) are increased after BMS treatment compared to Chon+. ALP staining (**A, B**) is reduced to a level of no visible ALP activity. Collagen type X staining is slightly increased at the edge of the aggregates after BMS treatment. **C, G, L, P** Chondrogenic control groups without TGFβ1 (Chon-). **D, H, M, Q** Test conditions without TGFβ1 after BMS treatment (Chon- w BMS). DMMB staining (**G, H**) and Collagen type **II** staining (**L, M**) are reduced after BMS treatment compared to Chon-. Collagen type X staining (**P, Q**) under both conditions is undetectable. Some single spots of ALP activity are visible in the control aggregates (**C**), but there is no ALP staining at all in the BMS group (**D**). Scale bar = 500 µm

Under hypertrophic conditions attenuation of hypertrophy by BMS depended on the time point of application during pellet culture. DMMB staining of hypertrophic control aggregates demonstrated the very pronounced hypertrophic phenotype with big, ballooned cells. Application of BMS in the late phase of chondrogenesis didn't cause any big morphological change, but hypertrophic cells were smaller than in the hypertrophic control group and were fewer at the center of the aggregate. However, there were distinct differences in the groups with early BMS treatment. Apart from single hypertrophic cells the aggregates had a very homogenous structure consisting of small chondrocytes and an ECM rich in GAGs.

ALP staining after late BMS treatment was reduced in the center and the outer zone of aggregates but still strong in the intermediate layer (FIGURE 15 B). Early BMS application decreased ALP activity even more, although there was still some ALP staining at the edge of the aggregates (FIGURE 15 C). Collagen II staining was unexpected strong in hypertrophic groups (FIGURE 15 G), as it is actually a chondrogenic marker, which physiologically decreases in reverse order to collagen type X content. This phenomenon may be founded on the effect of BMP4 in the proliferation medium to enhance hypertrophy. We assume that BMP4 initially boosts the entire synthesis activity of the chondrogenically differentiated MSCs including production of chondrogenic markers. Collagen type II concentration would probably decrease upon longer term duration.

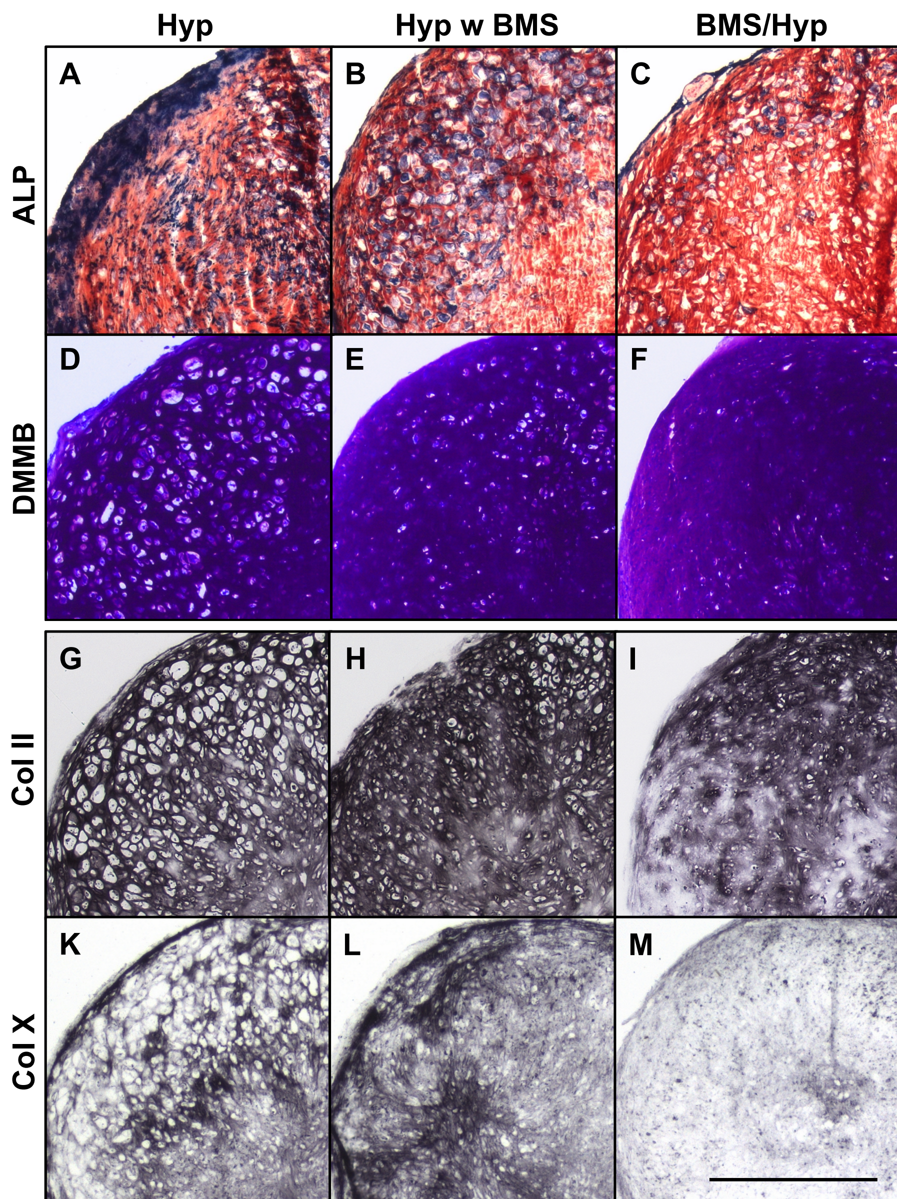


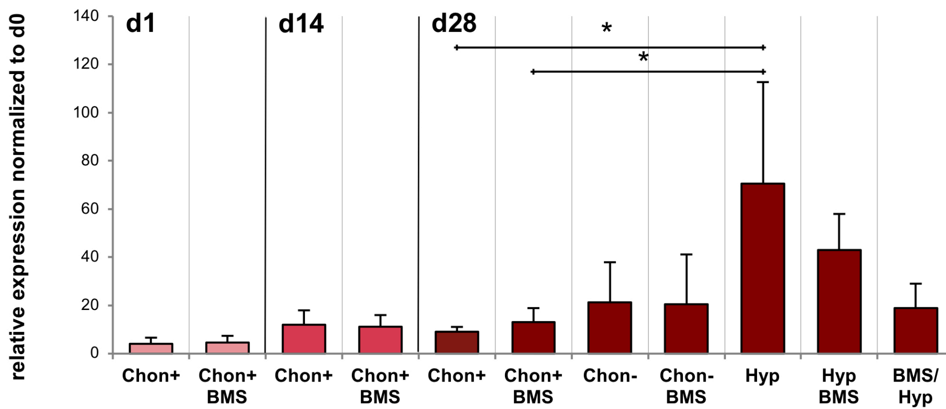
FIGURE 15 **Histological appearance of MSC aggregates on day 28 under hypertrophic conditions. A, D, G, K** Hypertrophic control groups (Hyp). **B, E, H, L** Hypertrophic aggregates after BMS treatment in late phase of differentiation (Hyp w BMS). **C, F, I, M** Hypertrophic aggregates after BMS treatment in early phase of differentiation (BMS/Hyp). There are remarkable changes in ALP (**A, B, C**), DMMB (**D, E, F**), collagen type II (**G, H, I**) and collagen type X staining (**K, L, M**) after late BMS treatment concerning volume and number of hypertrophic cells as well as expansion of hypertrophic markers (ALP, collagen type X). Early BMS treatment (**C, F, I, M**) leads to even greater changes including fewest hypertrophic cells, collagen type X content and ALP activity among the three hypertrophic conditions. Scale bar = 500 µm

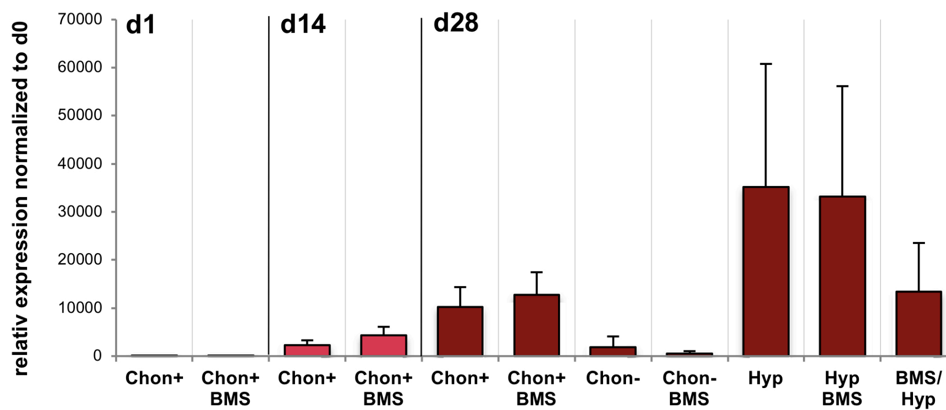
Immunostaining of the hypertrophic marker Collagen type X was strong in hypertrophic control groups and spread over almost the whole aggregate (FIGURE 15 K). In Groups with BMS treatment in the late phase of differentiation collagen type X staining was influenced slightly. It was focused on the edge of the aggregates and a little reduced in the center (FIGURE 15 L). However, in aggregates that had obtained BMS during the first two weeks of pellet culture collagen type X staining was reduced most showing just a slight grey color (FIGURE 15 M).

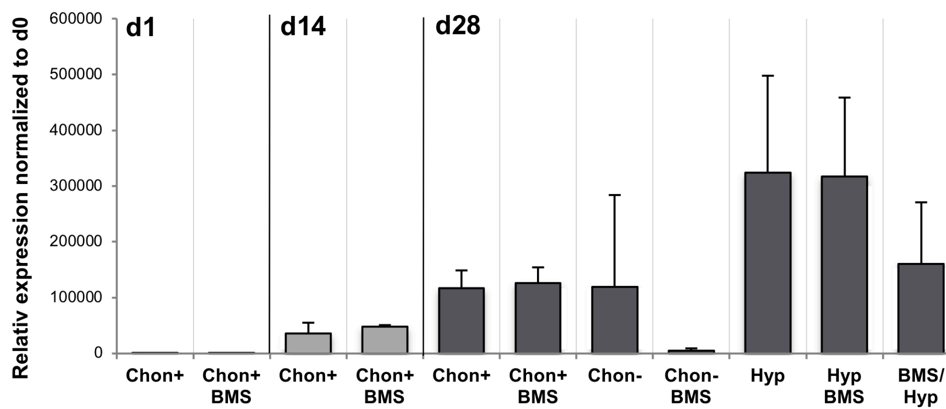
4.2.2 Gene expression analysis

To further investigate the effect of BMS493 treatment to MSCs chondrogenesis and hypertrophy we performed a gene expression analysis of the chondrogenic marker collagen type II, the hypertrophic markers collagen type X and MMP13 and the osteogenic marker collagen type I. Under chondrogenic conditions BMS treatment has no influence on the expression of MMP13 and collagen type X on day 28 compared to chondrogenic control groups. Equally there is no change in gene expression of the hypertrophic markers detected after BMS treatment in combination with withdrawal of TGFβ1. However, under hypertrophy enhancing conditions BMS treatment seams to decrease expression of MMP13 and collagen type X on day 28 especially after BMS application during early stage of differentiation (BMS/Hyp). Although there are no statistically significant differences between the hypertrophic groups probably based on the number of donors (n=3) gene expression of collagen type X in BMS/Hyp is on the same level as in the chondrogenic groups (Chon+, Chon+ BMS), which never the less is an indication for the pro-chondrogenic effect of BMS. Also, the differences in MMP13 expression between the hypertrophic groups on day 28 are not statistically significant. But there are significant differences between the hypertrophic control group (Hyp) and the two chondrogenic groups (Chon+, Chon+ BMS) but no significant differences between the chondrogenic groups and the two BMS treated hypertrophic groups (Hyp BMS, BMS/Hyp). This means that BMS treatment under hypertrophic conditions downregulated MMP13 gene expression as far as the differences to chondrogenic groups are no longer significant. Similar to histological analysis gene expression of collagen type II is unexpected high in hypertrophic groups, what we, as previously explained, attribute to the impact of BMP4. However, collagen type II gene expression in hypertrophic aggregates with early BMS treatment (BMS/Hyp) is on the same level with gene expression in chondrogenic aggregates (Chon+, Chon+ BMS), which is a sign for the favorable effect of BMS on MSCs chondrogenesis and attenuation of the effect of BMP4.

As expected, collagen type I expression is on the same low level under every condition and at every time point without significant differences. BMS treatment seems to have no regulating effect on collagen type I expression neither under chondrogenic nor hypertrophic conditions.







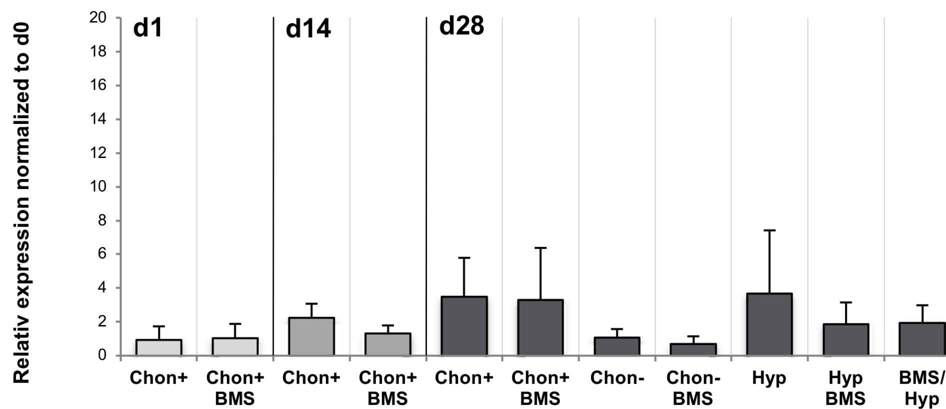


FIGURE 16
Gene expression analysis of the chondrogenic marker collagen type II, the osteogenic marker collagen type I and the hypertrophic markers, collagen type X and MMP13, in MSC aggregates after BMS treatment under chondrogenic, TGFβ1 free and hypertrophy enhancing conditions on days 1, 14 and 28 analyzed by real time PCR. normalized to day 0. n = 3 different donors.

4.2.3 Biochemical analysis

For biochemical analysis Sulfated GAG content of each pellet was determined and related to the DNA content as a quantitative differentiation marker. ALP activity in the medium supernatant was used as a quantitative marker for hypertrophy.

4.2.3.1 GAG content

DNA and GAG content were measured on day 1, day 14 and day 28. There was no significant difference in DNA content throughout all groups and time points (FIGURE 17 A), which is a sign for an equal number of cells and no increased rate of cell division or apoptosis under particular conditions. However, there were significant differences in GAG content relativized to DNA on day 28 (FIGURE 17 B). For example, GAG content was significantly reduced after withdrawal of TGFβ1 from day 14 of cell culture (Chon-) compared to the chondrogenic control group (Chon+). This shows similarly to histological and PCR results that chondral differentiation capacity of human MSCs is very low under TGFβ1 free conditions and BMS treatment (Chon- BMS) is not capable of improving that loss. Under hypertrophy enhancing conditions BMS treatment in the early phase of differentiation (BMS/Hyp) significantly decreased GAG content compared to the group with late BMS treatment (Hyp BMS). As a sign for increased synthesis activity the high GAG content in the hypertrophic group similar to the high collagen type II gene expression can probably be traced back to the effect of BMP4.

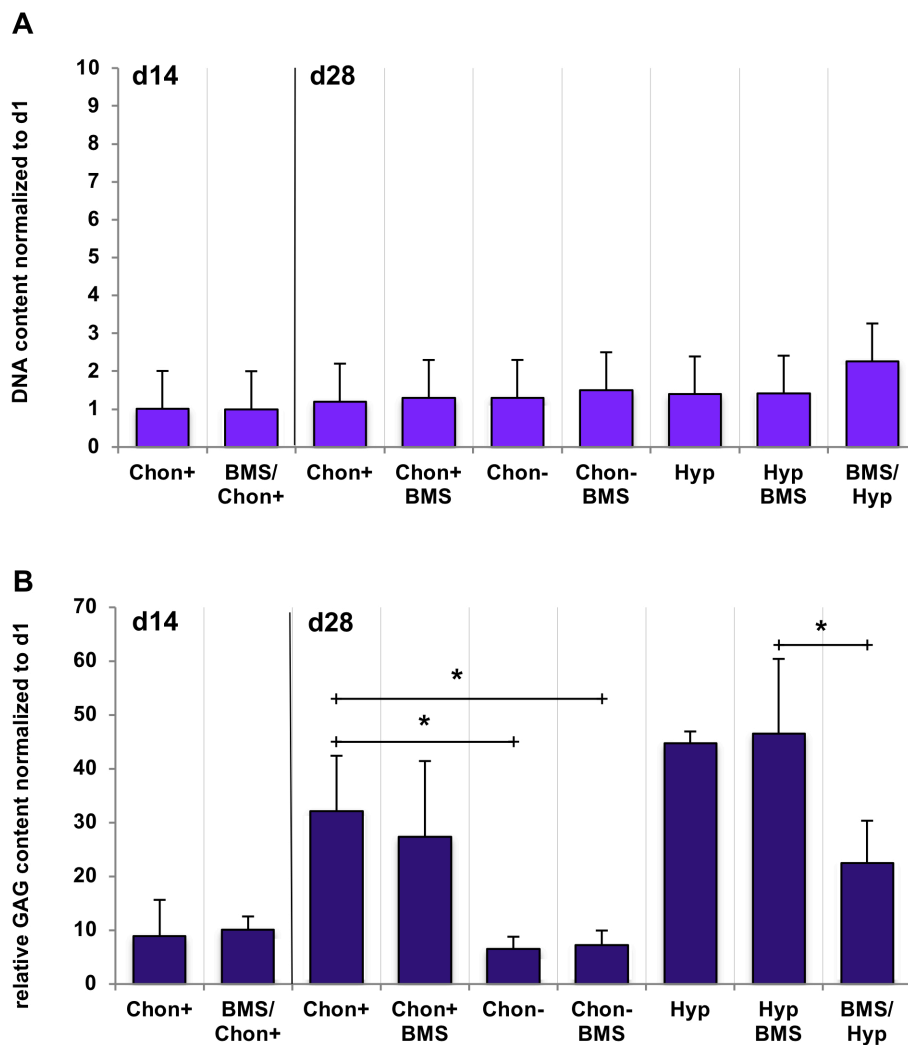


FIGURE 17
Biochemical analysis of MSC aggregates on day 14 and day 28. Data are normalized to those of day 1. A DNA content. **B** GAG content relative to DNA content. DNA content is equal throughout all groups and time points. GAG content is significantly decreased under TGFβ1 free conditions compared to chondrogenic control groups. Under hypertrophic conditions early BMS treatment effected a significant reduction in GAG content compared to the late BMS group.

4.2.3.2 ALP activity

ALP activity analysis in medium supernatant on day 28 of MSC pellet culture revealed that ALP activity was significantly higher in hypertrophic control aggregates (FIGURE 19 Hyp) compared to chondrogenic control aggregates (FIGURE 19 Chon+). These findings are in accordance with the histological results as alkaline phosphatase is a reliable biochemical marker for hypertrophy and in later stages for osteoblast activity. However, there are no significant differences in ALP activity between the chondrogenic control group and the hypertrophic groups treated with BMS, both early treatment group (FIGURE 19 BMS/Hyp) and late treatment group (FIGURE 19 Hyp w BMS). This means that BMS treatment under hypertrophy enhancing conditions independently from the time point of treatment had reduced ALP activity as far, that it is no longer significantly different from ALP activity under chondrogenic conditions. Thus, there is a distinct effect of BMS treatment to activity of the hypertrophic marker ALP.

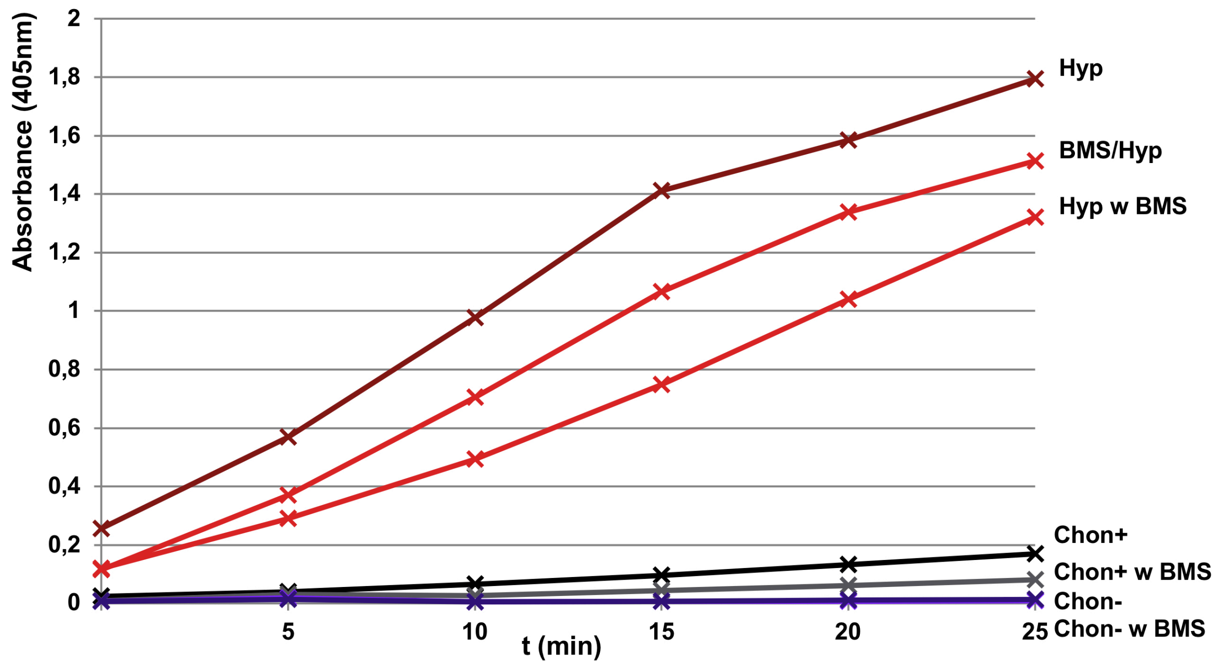


FIGURE 18 **Change of absorbance at 405nm in medium supernatant of day 28 MSC pellets over time. Graphs show the linear range of reaction. The gradients of the single graphs are directly proportional to the enzymatic activity in the associated groups.** ALP activity is highest in hypertrophic groups and very low in chondrogenic groups. There is nearly no ALP activity under TGFβ1 free conditions. n = 3

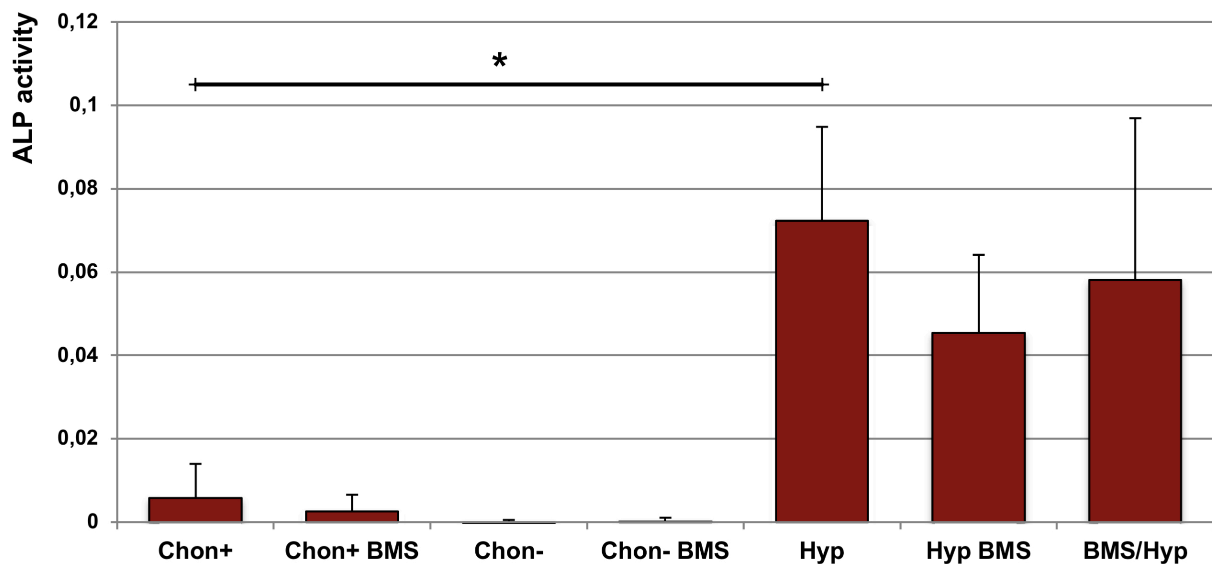


FIGURE 19 **ALP activity in the medium supernatant of day 28 MSC pellets.** ALP activity is significantly increased in the hypertrophic control group (Hyp) compared to the chondrogenic control group (Chon+). But there are no significant differences in ALP activity between the BMS treated hypertrophic groups and the chondrogenic control group. Thus, BMS treatment under hypertrophic conditions had reduced ALP activity as far as it is no longer significant higher to ALP activity under chondrogenic conditions. n = 3

5 Discussion

During skeletal development retinoids are important regulators of growth plate chondrogenesis [70], [71], [138]. RAR signaling is supposed to inhibit differentiation of pre-chondrogenic cells into chondroblasts [57], [58] and to promote chondrocyte maturation during endochondral ossification [56]. Thus, inhibition of RAR signaling with specific retinoid antagonists in order to suppress hypertrophic conversion and therefore support chondrogenic differentiation seems to be a promising way to solve current problems of *in vitro* MSC chondrogenesis. Nevertheless, retinoid acid signaling is complex and accompanied with several downstream pathways, which are modulating and mediating retinoid effects and have to be considered in conclusions about the potential effect of RAR antagonism.

In this study we investigated whether the modulation of the RAR pathway with a pan-RAR inverse agonist is capable of promoting or even inducing chondrogenesis of human MSCs *in vitro*. We used standard long term chondrogenic pellet culture to investigate the effect of the synthetic RAR inverse agonist BMS493 on chondrogenesis, matrix synthesis and cell phenotype of human MSCs *in vitro*. Additionally, we employed an established hypertrophy inducing media condition in order to unveil anti-hypertrophic influence of BMS493. Our data show that BMS is a promising attenuator of MSC hypertrophy and can promote TGFβ induced differentiation. However, BMS cannot replace TGFβ in a pro-chondrogenic environment.

BMS decreased expression, production and activity of hypertrophy related proteins such as MMP13, collagen type X and ALP. Interestingly, markers related to stable hyaline cartilage like collagen type II and glycosaminoglycans were also down-regulated by BMS under hypertrophic conditions. However, chondrogenic markers were less influenced by BMS than hypertrophic markers.

We chose *in vitro* pellet culture to study the influence of retinoid inhibition on chondrogenic differentiation of MSCs. Pellet culture is a suitable system for our purpose as it is not impaired by undefined soluble compounds and mimics the three-dimensional cell-cell contact. This biochemical interplay allows for improved cartilage formation as already shown by multiple working [35], [36], [39], [40]. Crucially, the serum-free differentiation medium is especially free of retinoids. Neither the

chondrogenic medium nor the hypertrophic medium contains any retinoids or precursor molecules, and mammalian cells are incapable of de novo retinoid synthesis [139].
On the other hand, this model does not represent the physiological situation during limp development that is characterized by the influence of retinoic acid especially in the late stage of chondrocyte maturation, when vessels spread into the cartilage template. In our study design tRA lacks as a competitive rival for BMS at RAR as we focus on the unimpaired effect of BMS. The presence of the full RAR agonist might interfere or at least diminish the BMS effect. Unliganded RAR acts pro-chondrogenically by basal repression of target gene transcription [73] and inverse agonists like BMS are capable of further reducing basal receptor activity in the absence of the physiological agonist [82].

5.1 Differentiation of MSC aggregates under chondrogenic and hypertrophy enhancing conditions

As MSCs are multipotent cells they are able to differentiate into a range of different mesenchymal tissues including cartilage. Though *in vitro* chondrogenically differentiating MSCs tend to undergo hypertrophy characterized by an increased cell size and expression of hypertrophic markers. The hypertrophic phenotype of chondrogenic differentiation can be significantly enhanced by special hypertrophy inducing medium containing BMP4 as described previously [42], [44]. To illustrate the anti-hypertrophic effect of BMS493 we used this hypertrophy enhancing model besides the standard chondrogenesis model, which includes addition of TGFβ1 and dexamethasone instead of BMP4. The rise of hypertrophy was clearly demonstrated by increased volume and swollen appearance of the chondrocytes, stronger ALP and collagen type X staining and higher expression of MMP13 in hypertrophic MSC aggregates. But also aggregates that were kept under chondrogenic conditions exhibited hypertrophic characteristics even though to a lower degree.
ALP staining was strong in hypertrophic MSC pellets but limited to small isolated spots and a thin ring around the aggregates under chondrogenic conditions. As described by Yoo *et al* there is a layer of flattened fibroblast like cells around the aggregates that did not differentiate chondrogenically and form a structure similar to the perichondrium [36]. Thus, we assume that the ALP positive frame around the chondrogenic MSC pellets more likely consists of fibroblast like cells than of hypertrophic chondrocytes.

Next to the histological findings ALP activity analysis exhibited clear results. ALP activity in the medium supernatant was significantly increased under hypertrophic conditions compared to chondrogenic control aggregates.
Collagen type X staining showed distinct differences between chondrogenic and hypertrophic groups. While chondrogenic aggregates featured only a slight staining, collage type X was clearly increased under hypertrophic conditions. However, in opposition to the histological findings there were no significant differences in collagen type X gene expression. This has been described in several other studies [40], [140], [141]. One possible explanation may be that post-transcriptional modulations effect these differences in final protein synthesis, which can be seen in immunohistochemical staining. On the other hand, gene expression of collagen type X might already have been decreased at this stage of hypertrophic conversion, while collagen type X protein is still traceable.
We investigated collagen type II gene expression and synthesis, as it is a typical chondrogenic marker, which physiologically decreases in inverse proportion to collagen type X content. But there were no statistically significant differences between chondrogenic and hypertrophic groups in collagen type II gene expression. Immunohistochemical collagen type II staining was strong under chondrogenic conditions as well as hypertrophy enhancing conditions. And collagen type II gene expression even tended to be higher in hypertrophic aggregates compared to chondrogenic control groups.
These unexpected findings in hypertrophic groups may be founded on the effect of BMP4 in the proliferation medium to enhance hypertrophy. We assume that BMP4 initially boosts the entire synthesis activity of the chondrocytes including production of chondrogenic markers. BMP4 as a bone-inductive protein is involved in chondrocyte hypertrophy and matrix degradation but there is also evidence for BMP4 to stimulate chondrogenic differentiation and cartilage ECM synthesis in an earlier step of chondrogenesis including expression of collagen type II [113]. Collagen type II expression and concentration would probably decrease in progress of further hypertrophic differentiation.
Only PCR analysis of the hypertrophic marker MMP13, which is involved in degradation of cartilage ECM proteins, revealed a significant higher gene expression level in hypertrophic groups compared to chondrogenic groups.

In summary we successfully enhanced the hypertrophy of chondrogenically differentiating MSCs by changing medium conditions, which was demonstrated by the histological phenotype, increased ALP activity and higher MMP13 gene expression. But we detected an unexpected high level of the chondrogenic marker collagen type II under BMP4 treatment. This factor has to be considered in the interpretation of our further results.

5.2 Attenuation of hypertrophy upon treatment with BMS493

Retinoid signaling plays a key role in chondrocytes maturation during limb skeletogenesis. As retinoids induce hypertrophy and mineralization and attenuate chondrogenic differentiation we assumed that inhibition of the RAR pathway by treatment with the RAR inverse agonist BMS493 would decrease or even prevent hypertrophy in chondrogenically differentiating MSCs. To investigate the effect of BMS treatment under different conditions we used a hypertrophy enhancing cell culture model as well as a standard *in vitro* chondrogenesis model with established chondrogenic medium containing TGFβ1. Additionally, we launched a test series under TGFβ1 free conditions. Under hypertrophy enhancing conditions BMS was applied during two different periods. One group obtained BMS during the early phase of chondrogenesis and a second one during the late phase.

5.2.1 BMS treatment under TGFβ1 free conditions

Our results indicate that MSCs did not fulfill an adequate chondrogenic differentiation under TGFβ1 free conditions. Collagen type II staining in TGFβ1 free aggregates was very weak compared to chondrogenic groups treated with TGFβ1 and collagen type X staining was not detectable at all. The weak DMMB staining and neutral red counterstaining in ALP aggregates indicate a small content of sulfated GAGs, which was supported by biochemical analysis. GAG content was significantly reduced after withdrawal of TGFβ1 compared to the chondrogenic control group. Thus, ECM synthesized in TGFβ1 free MSC pellets did not feature typical characteristics of cartilage ECM including high content of aggrecan and collagen type II.

The application of BMS after withdrawal of TGFβ1 had no positive impact on chondrogenesis, actually it seemed to inhibit chondrogenesis in a certain way based

on the weaker DMMB and collagen type II staining compared to the TGFβ1 free control group. BMS treatment did not effect a statistically significant increase in GAG content. Thus, these results disagree with the hypothesis, that BMS treatment allows a proper chondrogenesis in absence of TGFβ, and thereby don't support the findings of Wael *et al.* who showed induction of chondrogenic differentiation in hMSCs by treatment with an RAR antagonist in absence of growth factors [142].

5.2.2 BMS treatment under chondrogenic conditions

Histological analysis revealed some differences between chondrogenic control groups and BMS treated test groups.
MSC aggregates treated with BMS were free of ALP positive cells, whereas chondrogenic control aggregates exhibited ALP staining in their outer zone.
DMMB staining was a bit stronger in the BMS group, which is a sign for a higher content of GAGs in the ECM. And chondrogenic aggregates with BMS treatment showed a higher content of collagen type II compared to chondrogenic control groups. These histological findings are evidence for the pro-chondrogenic effect of BMS. However, biochemical and gene expression analysis did not show significant differences between BMS treated aggregates and control aggregates under chondrogenic conditions.
In summary, effects of BMS treatment on hypertrophy under chondrogenic conditions were little and restricted to the histological level because the hypertrophic phenotype in this phase of differentiation has not been strong enough yet to allow great differences. This leads us to our most relevant groups. Under hypertrophic conditions we were able to achieve a distinct attenuation of hypertrophy by BMS treatment.

5.2.3 BMS treatment under hypertrophy enhancing conditions

The efficiency of BMS treatment under hypertrophic conditions was dependent on the time point of application in pellet culture. Starting with the histological morphology BMS treatment in the late phase of chondrogenesis only produced little reduction in volume and number of hypertrophic cells whereas the group with early BMS treatment showed distinct morphological differences. Apart from single hypertrophic cells the aggregates had a very homogenous structure consisting of small chondrocytes. Staining of the

hypertrophic markers, ALP and collagen type X, was reduced in both, early and late BMS treatment groups.
However, aggregates that had obtained BMS during the first two weeks of pellet culture showed the best histological results.
Collagen type II staining and gene expression was on an unexpected high level in hypertrophic groups, what we as previously explained attribute to the impact of BMP4. However, collagen type II gene expression in hypertrophic aggregates with early BMS treatment is down-regulated to the same level as gene expression in chondrogenic aggregates, which may be a sign for the pro-chondrogenic effect of BMS on MSCs and the potential attenuation of the effect of BMP4 under BMS treatment. Several studies from different medical fields indicate that RAR and BMP signaling are closely associated and cooperate on various levels: Apart from direct retinoid effects on chondrocyte maturation through activation of RARs, retinoic acid furthermore seems to stimulate genes encoding BMPs, which leads to an indirect induction of collagen type X expression in pre-hypertrophic chondrocytes [143], [144]. Leaving the field of orthopedics, BMP and RA reveal more synergistic effects. Within oncology retinoids are clinically used in therapy of epithelial cancer and promyelocytic leukemia to initiate apoptosis in tumor cells [145], [146]. Müller *et al.* recently showed that retinoic acid and BMP synergistically induce apoptosis in retinoblastoma cell lines. BMP4 treatment increased the gene expression of specific RAR and RXR subtypes [147]. With regard to these enhancing interactions between the RAR and the BMP pathway it appears reasonable that inhibition of RAR signaling with BMS has negative influence on BMP4 effects.
Gene expression of the hypertrophic markers MMP13 and collagen type X was reduced by BMS treatment especially by BMS application in the early stage of differentiation.
Biochemical analysis revealed a statistically significant difference between early BMS treatment and late BMS treatment under hypertrophy enhancing conditions. BMS treatment in the early phase of differentiation significantly decreased GAG content compared to the group with late BMS treatment.
As a sign for increased synthesis activity the high GAG content in hypertrophic group similar to the high collagen type II gene expression can be probably traced back to the effect of BMP4. Consequently, the significant reduction of GAG content under BMS

treatment in early phase of differentiation may demonstrate an attenuation of this BMP effect by BMS.
We found statistically significantly higher ALP activity in the hypertrophic control group compared to the chondrogenic control group. But BMS treatment in both, early and late phase of differentiation, reduced ALP activity under hypertrophy enhancing conditions down to the range of the chondrogenic groups.

Although *in vitro* results are promising there will be some limitations for the *in vivo* use of BMS. Indeed, retinoids are clinically used for a number of therapeutic indications including cancer, psoriasis, acne and diabetes but they can lead to severe side effects especially through systemic application. Thus, the topical application by intraarticular injection would be favorable. For the therapy of osteoarthritis, Yin *et al.* developed a drug delivery system especially for BMS using an engineered cartilage oligomeric matrix protein coiled-coil protein to encapsulate and protect the hydrophobic and unstable BMS molecule [148].
Furthermore, there are novel, atypical drugs that might be an alternative to BMS *in vivo*. Busby *et al.* presented the first non-acid, non-retinoid direct modulator of the RAR superfamily that acts as a pan-RAR inverse agonist like BMS but features an improved toxicity and pharmacokinetic profile over classical retinoids [149]. However, their efficiency in chondrogenesis protocols has to be further investigated.

In this study, we validated that chondrogenically differentiated human MSCs in 3D aggregate can produce features of hypertrophic conversion. Hypertrophic media challenge furthermore enhances hypertrophy. However, inclusion of the RAR inverse agonist BMS attenuated these hypertrophic changes, which may be useful in producing stable engineered tissue for cartilage regeneration. Specifically, when BMS was administered under hypertrophic conditions, a decrease in cell size, ALP activity and gene expression of hypertrophic markers could be observed. Our experiments revealed the early phase of chondrogenesis as the best period for the application of BMS in order to attenuate hypertrophy. Based on these findings, current studies are exploring the dose of BMS to most efficaciously prevent hypertrophic changes, as well as the duration of its effect and delivery in a therapeutic setting. Further, we are investigating the pathways through which BMS493 exerts its effects.

6 Conclusion

In our study we demonstrated that inclusion of the RAR inverse agonist BMS493 during *in vitro* chondrogenesis of hMSCs can attenuate hypertrophic changes, which may be useful in producing stable engineered tissue for cartilage regeneration. Specifically, when BMS was administered under hypertrophic conditions, a decrease in cell size, ALP activity and gene expression of hypertrophic markers is observed. Our experiments reveal the early phase of chondrogenesis as the best period for the application of BMS in order to attenuate hypertrophy. Moreover, our findings indicate that the chondrogenic effect of BMS is dependent on the presence of TGFβ1 and that BMS exerts its' effect at least partially through interaction with BMP signaling. Thus, there are several connections between BMP and the TGFβ-Superfamily. However, their exact relationship during chondrogenesis deserves further investigation.
Beside the very convincing histological outcome our results hardly reached statistical significance due to the low number of donors, even if there is a clearly visible tendency regarding the pro-chondrogenic effect of BMS. Further studies are planned to confirm the current results.

7 References

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8 List of Abbreviations

ACL	Anterior crucial ligament
ALP	Alkaline phosphatase
BAMBI	BMP and activins membrane-bound inhibitor
BMP	Bone morphogenic protein
BMPR	Bone morphogenic protein receptor
CBFA1	Core binding factor subunit α1
CoA	Co-activator
CoR	Co-repressor
CRABP	Cellular retinoic acid binding protein
DMMB	Dimethylmethylenblue
ECM	Extracellular matrix
GAG	Glycosaminoglycan
GDF	Growth and differentiation factor
GSK	Glycogen synthase kinase
hMSC	human mesenchymal stem cell
ICRS	International cartilage regeneration & joint preservation society
IGF	Insulin-like growth factor
IHH	Indian hedgehog homolog
Lef/TCF	Lymphoid enhancer factor/T cell factor
LRP	Lipoprotein receptor related protein
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
PDGF	Platelet-derived growth factor
PG	Proteoglycan
PTHrP	Parathyroid hormone related environment
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinoid binding protein
RDH	retinol dehydrogenase
Runx2	Runt-related transcription factor 2

RXR	Retinoic X receptor
SMAD	Sma and Mad related protein
SOX9	*SRY related HMG box 9*
STRAT6	“stimulated by retinoic acid” RBP receptor/Vit. A transporter
TGFβ	Transforming growth factor β
tRA	all-trans retinoic acid

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