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Extracellular Matrix Based Materials for Tissue Engineering

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Abstract
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The extracellular matrix is (ECM) is a network of large, structural proteins and polysaccharides, important for cellular behavior, tissue development and maintenance. Present thesis describes work exploring ECM as scaffolds for tissue engineering by manipulating cells cultured *in vitro* or by influencing ECM expression *in vivo*. By culturing cells on polymer meshes under dynamic culture conditions, deposition of a complex ECM could be achieved, but with low yields. Since the major part of synthesized ECM diffused into the medium the rate limiting step of deposition was investigated. This quantitative analysis showed that the real rate limiting factor is the low proportion of new proteins which are deposited as functional ECM. It is suggested that cells are pre-embedded in for example collagen gels to increase the steric retention and hence functional deposition.

The possibility to induce endogenous ECM formation and tissue regeneration by implantation of growth factors in a carrier material was investigated. Bone morphogenetic protein-2 (BMP-2) is a growth factor known to be involved in growth and differentiation of bone and cartilage tissue. The BMP-2 processing and secretion was examined in two cell systems representing endochondral (chondrocytes) and intramembranous (mesenchymal stem cells) bone formation. It was discovered that chondrocytes are more efficient in producing BMP-2 compared to MSC. The role of the antagonist noggin was also investigated and was found to affect the stability of BMP-2 and modulate its effect. Finally, an injectable gel of the ECM component hyaluronan has been evaluated as delivery vehicle in cartilage regeneration. The hyaluronan hydrogel system showed promising results as a versatile biomaterial for cartilage regeneration, could easily be placed intraarticulary and can be used for both cell based and cell free therapies.

Keywords: Extracellular matrix, collagen synthesis, bioreactor, cell culture, bone morphogenetic protein-2, noggin, hyaluronan, cartilage

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Till Farmor

List of Papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I **Extracellular matrix-polymer hybrid materials produced in a pulsed-flow bioreactor system**
Aulin, C., Foroughi, F., Brown, R. and Hilborn, J
Journal of Tissue engineering and regenerative medicine, **2009**, 3(3):188–195

II **Bulk collagen incorporation rates into knitted stiff fibre polymer in tissue engineered scaffolds: The rate limiting step**
Foroughi, F., Aibibu, D., Aulin, C., Hilborn, J. and Brown, R.
Journal of Tissue engineering and regenerative medicine, **2008,** 2(8):507-514

III **Comparative studies on BMP-2 processing and secretion in chondrocytes and mesenchymal cells and the effects of noggin**
Aulin, C., Hilborn, J., Lyons, K. M. and Engstrand, T.
Submitted manuscript

IV **Evaluation of an injectable hyaluronan hydrogel for cartilage regeneration**
Aulin, C., Bergman, K., Hedenqvist, P., Hilborn, J., Jensen-Waern, M. and Engstrand, T.
Manuscript

Papers not included in this thesis

V **Polarized protein membrane for high cell seeding efficiency**
Atthoff, B., Aulin, C., Adelöw, C. and Hilborn, J.
Journal of Biomedical Materials Research: Part B, **2007,** 83B:472-480

VI **In vitro degradation and in vivo biocompatibility study of a new linear poly(urethane urea)**
Asplund, B., Aulin, C., Bowden, T., Eriksson, N., Mathisen, T., Bjursten, L-M. and Hilborn, J.
Journal of Biomedical Materials Research: Part B, **2008**, 86B(1):45-55

VII **A poly(lactic acid-co-caprolactone)-collagen hybrid for tissue engineering applications**
Ananta, M., Aulin, C., Hilborn, J., Aibibu, D., Houis, S., Brown, R.A., Mudera, V.
*Tissue Engineering, Part A***, 2009,** 15(7):1667-1675

VIII **Ectopic induction of the tendon-bone interface**
Bergman, K., Docherty-Skogh, A., Aulin, C., Ossipov, D., Hilborn, J., Bowden, T. and Engstrand, T.
Submitted manuscript

My contributions to the papers in this thesis

I. I contributed to the design of the study, performed the main part of the experiments and wrote the manuscript.

II. I contributed to the design of the study, performed part of the experiments and participated in the discussion of the results.

III. I contributed to the design of the study, performed part of the experiments and wrote a major part of the manuscript.

IV. I contributed to the design of the study, performed the experiments, except surgical procedures and histological preparations and wrote the manuscript.

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Abbreviations

ALP	Alkaline phosphatase
BMP-2	Bone morphogenetic protein-2
DMEM	Dulbecco's modified Eagle's medium
DMMB	1,9-dimethylmethylene blue
ECM	Extracellular matrix
ER	Endoplasmatic reticulum
GAG	Glycosaminoglycan
H&E	Hematoxylin and Eosin
HPLC	High performance liquid chromatography
Hyp	Hydroxyproline
kDa	kilo Dalton
MSC	Mesenchymal stem cells
MTT	Thiazolyl Blue Tetrazolium Bromide
ORF	Open reading frame
PET	Poly(ethylene terephtalate)
PGA	Poly(glycolic acid)
PLLA	Poly(L-lactic acid)
SEM	Scanning electron microscopy
SMAD	Sma mothers against decapentaplegic
TGF-β	Transforming growth factor beta

Scope of thesis

Extracellular matrix (ECM) is the main constituent in connective tissues such as skin, cartilage and bone and also a key player for both tissue development and tissue maintenance. ECM is important for structure and mechanical strength of tissues, as well as playing an important role in cell signaling and tissue homeostasis. The ECM and its function has been a core aspect in this work as we have sought to design ECM based materials by influencing the behavior of cells *in vitro* as well as by regenerating ECM rich tissues *in vivo*.

In paper I and II, we set up a system where we could design extracellular matrix based materials for soft tissue regeneration by manipulating cells seeded on a polymer mesh under dynamic culture conditions. By combining the mechanical properties of a polymer mesh with the biological action of deposited ECM proteins we could produce materials with biomimetic composition and complexity. The dynamic culture conditions increased the ECM deposition but the levels were lower than target tissue contents. The limiting factor was the ability to retain synthesized protein for ECM assembly on the mesh.

In parallel we were also looking into regeneration of cartilage and bone. Here a different strategy was applied, which involved affecting endogenous cells with growth factors that were either produced by genetically engineered cells or from a recombinant source delivered to the damaged site.

In paper III, we investigated the intricate interplay between the bone promoting growth factor bone morphogenetic protein-2 (BMP-2) and its antagonist noggin to gain knowledge of how to produce and use BMP-2 to regenerate bone tissue in a more efficient way.

In paper IV, a hyaluronan hydrogel carrier developed in our lab, previously successfully used in bone tissue engineering, was explored for cartilage regeneration. We demonstrated that the hyaluronan hydrogel could sustain chondrocytes and mesenchymal stem cells *in vitro* and deposit a glycosaminoglycan rich cartilage ECM. Furthermore, a pilot study of cartilage regeneration in knee joint defects in rabbits was performed.

In summary this thesis has focused on the following key points:

- *in vitro* ECM deposition onto polymer materials
- BMP-2 expression and regulation
- BMP-2 induced cartilage regeneration with an injectable hyaluronan hydrogel carrier

1. Introduction

1.1 Tissue engineering and regenerative medicine

In the field of tissue engineering and regenerative medicine, new materials are developed to trigger regeneration of damaged tissue in coordination with cellular responses that should function in a clinical setting. Typically, it involves transplantation of viable cells or tissue-inducing substances together with a carrier material to a damaged site in the body.[1] The components are chosen based on the tissue to be restored, in order to direct the body into forming new, functional tissue (Figure 1.1).

As a topic of study, tissue engineering is interesting for several reasons. There is an innate human interest in the field. The ultimate goal is to develop new therapies for disorders that have proven difficult or impossible to treat successfully with the existing tools of medicine. This goal is somewhat provocative by its science-fiction like vision of man-made, living spare parts for the human body. At the same time it is appealing due to the fact that human lives are lost because of shortage of organ donors. Tissue engineering is highly interdisciplinary, meaning scientists have to face the technical challenges in many subdisciplines, not only from engineering but also science and clinical medicine. To be successful, integration of several very different kinds of knowledge and ways of thinking is necessary.

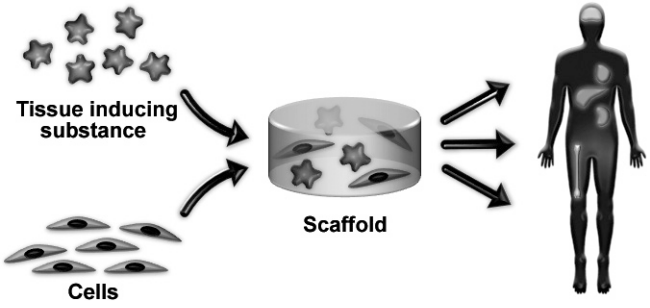

Figure 1.1 The principles of tissue engineering involve the delivery of cells and tissue inducing substances with a carrier material for the purpose of restoring lost tissue function. Reprinted from Bergman (2008).[2]

What can we learn from nature?

Tissue formation and regeneration occur in the human body throughout the entire life time, from embryogenesis to wound closure. These changes are closely associated with cell migration and proliferation as well as ECM deposition and remodeling.[3] During tissue development, matrix deposition and organization play an important role in guiding cells in migration and differentiation and an ECM is present already from the two-cell stage in mammalian embryos.[4] ECM is also very important in wound healing. Within the first phases of the wound repair process, tissue formation is characterized by migration of epithelial cells, which deposit extensive amount of ECM. Later phases include matrix remodeling and transformation of initial granulation tissue into connective tissue.[5]

The ECM has profound structural and biological effects on developmental processes, and formation and transformation of ECM is crucial to tissue regeneration and function. These characteristics of the intact ECM make it an excellent choice of scaffold for tissue regeneration and the ECM has been an inspiration throughout the course of this work, both in producing it *in vitro* or by formation *in vivo*.

During embryonic development, one single cell, the fertilized egg, develops into a complete organism consisting if several types of specific, diverse tissues. These events are guided by chemical, physical and mechanical cues in a precise time and space dependent row of events that eventually leads to an entire organism.[6] (Figure 1.2) We know to some extent about various events but a large part is still unknown and may also be a clue to successful tissue engineering and regeneration of tissues.

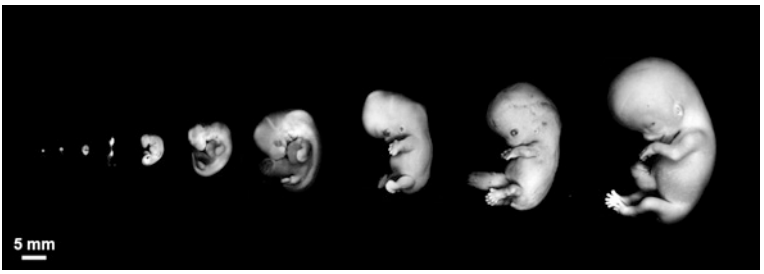

Figure 1.2 During embryonic development one single cell develops into a complete organism. These events are guided by chemical, physical and mechanical cues. The scaffolding needed for tissue growth in three dimensions is solved by the cells creating their own environment in form of ECM! (Modified from M.A. Hill, 2004)

Growth factors are a group of molecules capable of stimulating cellular growth, proliferation and differentiation. Growth factors are important for regulating a variety of cellular processes, both in fetal and adult individuals. For instance are vascular endothelial growth factor and basic fibroblast

growth factor involved in blood vessel formation[7], epidermal growth factor is involved in proliferation and cell growth of the epithelium[8] while bone morphogenetic proteins (BMPs) are involved in bone and cartilage formation[9]. Research regarding the cellular response to these types of molecules is common in the field of developmental biology and this knowledge is also useful when designing materials and systems to trigger regeneration. Bone and cartilage regeneration and underlying mechanisms for bone development, and in particular the bone and cartilage inducing growth factor BMP-2 and its antagonist noggin, will be discussed more thoroughly in a later section, since these played an important role in present investigations.

1.2 The extracellular matrix

ECM is the major constituent of multicellular organisms. It is composed of large structural proteins, glycosaminoglycans and proteoglycans that are secreted and assembled locally into an organized network (Figure 1.3). Together with cells they define the structure and properties of different tissues in the body. Connective tissues such as cartilage and bone consists of relatively few cells embedded in an extensive ECM, while parenchymal tissue like the liver almost exclusively consists of cells.

Previously, the role of the ECM was considered only structural, but we now know that it also plays an important role in cell signaling and tissue homeostasis.[10] The difference in ECM composition is providing the tissues separate functions by interactions between matrix molecules, growth factors and cell surface receptors, which mediate adhesion of cells to ECM components. For example, cartilage ECM is rich in collagen II and proteoglycans for resisting compression, basement membrane ECM is enriched in laminin and non-fibrillar collagen forming a separating interface between different tissue layers while in the kidney the ECM acts as a molecular filter.[11-13]

The ECM consists of structural and functional macromolecules, where collagen is the most abundant. There are more than 20 genetically different types of collagens identified so far. They are all based on the same structure; a right-handed triple helix made of three individual collagen chains wounded around each other.[14] Collagen type I is the primary structural protein in mammalian tissue and is ubiquitous across the animal and plant kingdom. It is the subject of many investigations and has been well characterized.[15] Fibrillar collagens provide the tissue with stability and tensile strength.

Elastin is another structural protein, which can be found in tissues that have the ability of stretching such as skin, bladder and blood vessels. By permitting deformation and recoil of tissue, elastin is providing structural integrity in response to mechanical stress. This elastic function is complementary to that of collagen fibers, which provide rigidity and tensile strength.[16, 17] Collagen and elastin may be considered as the main contribu-

tors of the mechanical properties of ECM. The quantity of elastic fibers and collagen fibers results from an equilibrium between the processes of synthesis and degradation, regulated by a number of proteases.[18]

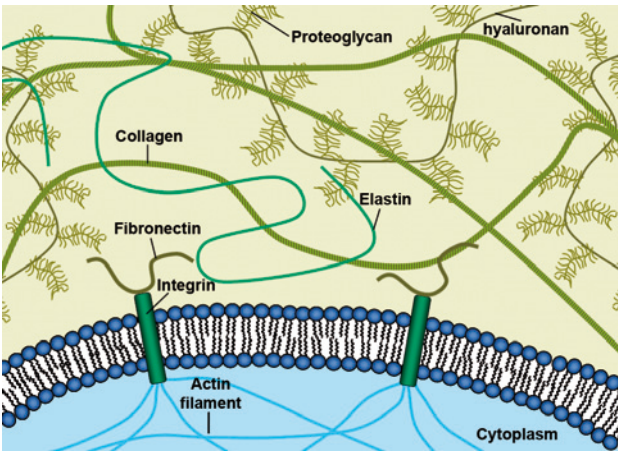

Figure 1.3 The extracellular matrix is composed of different macromolecules giving tissues their strength and shape. It consists of large structural proteins and polysaccharides important for mechanical properties as well as playing an important role in cell signaling and tissue homeostasis.

Collagens and elastin are supported in their biomechanical functions by the glycosaminoglycans (GAGs). GAGs are providing the gel-like properties of the ECM and in most cases they are components of proteoglycans. Proteoglycans consist of bottle brush structured GAGs grafted on a protein core. GAGs are strongly anionic polymers that absorb water, thus providing compressive strength to the tissue. GAGs are involved in regulation of water uptake, sequestering of growth factors and cell migration.[19] Hyaluronic acid is a special type of non-sulphated GAG which contributes to frictional resistance against interstitial fluid flow. It is an important lubricant in joints, and is often used in cartilage tissue engineering as a scaffold component.[20-22]

Proteins like fibronectin and laminin are responsible for cell adhesion, and can be regarded as the “ECM glue”. Fibronectin is the second most abundant protein in ECM, after collagen, and is a large glycoprotein with different binding domains. There are two types of fibronectin; the soluble form found in blood and the insoluble form found in ECM.[23, 24] Laminin is found in the basement membrane, an ECM structure separating the epithelium from underlying layers of connective tissue and muscle. It is involved in cell differentiation, migration and proliferation and plays a critical role in angiogenesis.[24-26]

Many ECM proteins self assemble outside the cell into structural scaffolds whose composition, supramolecular organization and biomechanical properties are adjusted to tissue functions. Their biosynthesis is often quite intricate and requires numerous and specific post-translational modifications which can occur both intra- and extracellulary.[27, 28] This complexity is making it hard to mimic the ECM synthetically. Recombinant production of ECM proteins has been achieved, but the hierarchical order and multifunctionality of a complete ECM is still far from being realized.[29] In present work, we have chosen a combined strategy; investigating the possibility to manufacture ECM based materials *in vitro*, by culturing cells that deposit their ECM on a polymer scaffold. We hypothesized to produce safe, reproducible materials by knowing the producing cell line, as well as gain control over the deposited ECM in terms of alignment and orientation to the scaffold template.

1.3 The use of scaffolds in tissue engineering

Scaffold properties

When designing materials for tissue engineering, guidance and inspiration drawn from ECM would be beneficial. In transplantation of viable cells or tissue-inducing substances, there is a need of a carrier to position and keep the transplanted factors in place. These carriers are often referred to as scaffolds, and should function as a temporary artificial ECM during regeneration.[30]

Scaffolds aim to mimic or enhance many ECM functions, in addition to complying with practical handling of grafts and implants. Scaffolds should have adequate mechanical, physical and chemical properties and preferably biological too in addition to being non-toxic and non-immunologic. The list can be made long and trade-offs are a prerequisite. Some important materials properties are listed in table 1.1.

Materials used today are hydrogels, meshes, sponges and electrospun materials, as well as ceramics and metallic compounds.[31] Polymers are important material candidates for scaffolds, since they can be tailored to have certain desired properties, including mechanical properties, three dimensional shape, biocompatibility and degradability.[32, 33] Polymers of both synthetic and natural origin and their combinations are available.

The most important feature of scaffolds is their possibility to recreate functional tissue. The ability to regenerate functional tissue is size and material dependable, and when the criteria are not met, non-functional tissue, i.e. collagen type I rich scar tissue, is obtained.[34] Scar tissue is formed in the body as a response to a defect being too large to be healed in time. However, this tissue is nonfunctional and prevents the normal function of the tissue. By

applying a temporary scaffold to the defect, the invasion of scar tissue could be hindered and would preferably trigger healing where it would otherwise fail.

Table 1.1. Important design properties for biomaterials

Property	Description
Porosity	Provides nutrient diffusion and allows cell migration
Mechanical properties	Modulus should match target tissue to avoid shear stress and fibrotic encapsulation. Material should be able to endure tensile stress, compression and other forces at the site of implantation.
Degradability	Degradation rate must match the rate of new tissue formation. No toxic byproducts should be formed.
Surface chemistry	Should be tailored to promote or prevent protein adsorption and cell attachment.
Surface topography	Important for cell adhesion and tissue integration via mechanical interlocking.
Non toxic	Both bulk material and degradation products must be non toxic, in short and long term. The material should not invoke immunogenic responses or show signs of genotoxicicty.
Biological activity	Could be an inherent material property containment of (non-bound) tissue inducing components.

Natural scaffolds

Many materials used in tissue engineering today are based on decellularized tissues or purified ECM components, including collagen type I sponges[35, 36], decellularized dermis[37, 38], Matrigel®[39] and small intestine submucosa[40]. Their main advantages are their biological properties and their degradability. They provide cell adhesion sites, biochemical information and morphological cues that are hard to mimic synthetically.[41] However, their mechanical properties are often poor and design possibilities are restricted without altering the biological properties. Other problems are purification processes that are extensive and tedious, the removal of traces of potentially pathogen transmitting residues and batch to batch variation depending of age and gender of donor. To avoid some of these problems, and to biochemically design natural ECM, we attempted to use cells or cell lines under controlled culture conditions to deposit a complete ECM, not just one or more components. However, cells need a substrate to grow on, and synthetic polymers are a well known type of materials for these purposes (Figure 1.4).

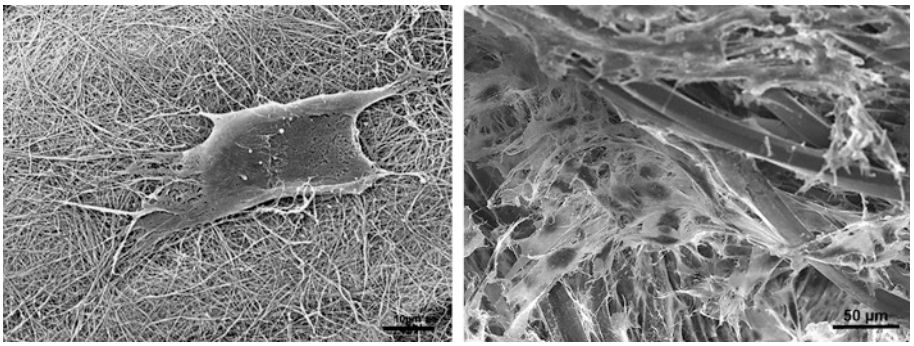

Figure 1.4 Cell on a collagen scaffold (left) and cells cultured in a polymer mesh scaffold (right).

For cartilage regeneration the use of the ECM component hyaluronic acid has several advantages. Cartilage tissue is rich in hyaluronic acid, making the polysaccharide a natural environment for chondrocytes. It has also been shown to play important roles in regulation of cellular differentiation and cartilage maintenance.[42] Hyaluronic acid is a widely used biomaterial which has been employed for various areas such as bone regeneration[43], eye surgery[44] and wound healing[45]. The presence of carboxylic acids on the backbone provides sites for chemical modification which can alter its mechanical properties and decrease the degradation rate *in vivo*.[46] These are factors we took advantage of in paper IV, investigating the possibility of cartilage regeneration using a hyaluronic acid hydrogel as carrier material.

Synthetic scaffolds

The development of new methods and synthetic routes has led to better materials with functional biomimetic structures. Biocompatible scaffolds include synthetic polymers such as poly(glycolic acid)[47], poly(lactic acid)[48], poly(ethylene glycol)[49] and poly (vinyl alcohol)[50]. They have been extensively explored and are biocompatible and easily modified to prepare materials of different properties, for instance hydrogels, meshes and foams. Once synthesized, polymers can be processed or modified further to provide tailored conditions for cell culture. Important factors for cell growth are porosity, mechanical properties and surface chemistry.[30]

Polymers used in this work were knitted meshes, which provide compliant, three dimensional, porous scaffolds and hydrogels based on the natural polysaccharide hyaluronan (Figure 1.5). Knitted structures have a large specific surface area for cell adhesion and give enough space for cells to grow and develop an ECM environment. Poly(ethylene terephtalate) (PET) is a non-degradable, biocompatible polymer used for hernia repair and as scaffolds for cell growth.[51]

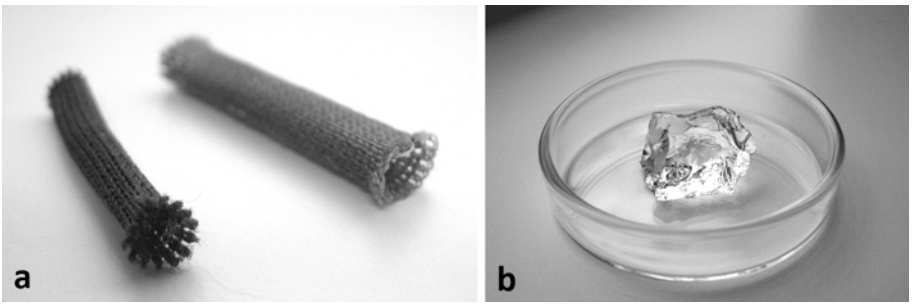

Figure 1.5 Scaffolds used in this work were (a) warp knitted polymer meshes of PET (Paper I and II) and PGA (Paper II) and (b) a hydrogel based on the ECM component hyaluronan (Paper IV).

Weft, knitted PET scaffolds was used as a model system to decide culture conditions and cell behavior in mechanically stimulated culture, without affecting the mechanical performance by degradation of the polymer. Biodegradable polymers are more attractive to use, since the scaffold should function as a temporary support for cells as new tissue is formed. Poly(glycolic acid) (PGA) and poly(L-lactic acid) (PLLA) are FDA approved degradable polyesters, used mainly for sutures and orthopedic pins.[52] PGA is highly crystalline and degrades within 2-4 weeks, while PLLA is degraded slower, over several months. To adapt and improve materials properties, copolymers of PGA and PLLA is often applied. In copolymers the high crystallinity is rapidly lost and these morphological changes lead to an in increase in the rate of hydration and hydrolysis meaning copolymers degrade more rapidly than either PGA or PLA. Thus, degradation rate of the copolymers can be tuned by changing the PGA:PLLA ratio.[53]

Mechanical stimulation as a morphological cue

Not only the inherent properties of the materials are important, also external environmental cues applied to the cell-material system need to be considered, such as chemical factors and mechanical forces. Large effort has been made in understanding how hormones, growth factors and cytokines regulate the expression of ECM genes, while changes in tissue structure and mechanics have been comparatively neglected. It is well known that cells do respond to mechanical stimulation, although the pathway from a mechanical stimulus to altered ECM gene transcription is not well understood.[54-57] To guide the remodelling process it is essential to understand how cells respond to the matrix, to each other, to imposed mechanical forces as well as to secreted factors as they reorganize themselves and the matrix. The ECM is directly connected to the cytoskeleton and can mediate mechanical signals into the cell via cell receptors and transmembrane proteins, of which the integrin

family is one of the most important. Molecules that mediate mechanotransduction may therefore represent future targets for therapeutics in a variety of diseases. One example is different cancers since many cancer cells exhibit particular biomechanical properties.[58]

A major problem when designing scaffolds is to determine how to induce the cells to align, orient and interact amongst themselves and remodel the matrix in ways that yield a tissue equivalent that mimics the mechanical properties of the tissue targeted for replacement. By culturing cells in a bioreactor that offers specific mechanical forces, such as stretch, pressure or shear forces, cells can be guided into specific structural and functional characteristics.[59-61] Several types of bioreactors have been developed for controlled *in vitro* tissue engineering, including rotating wall vessels, perfusion bioreactors and bioreactors that apply controlled mechanical forces.[55, 57, 59, 62-65] In present work a bioreactor system from Tissue Works Ltd, Manchester or a development thereof was used, consisting of a closed chamber with regulated temperature and pressure, applying mechanical stimulation by peristaltic pumping.

1.5 BMP-2 in bone and cartilage regeneration

Endochondral and intramembranous bone formation

The skeleton of our body consists of an abundant mineralized ECM containing bone forming and bone resorbing cells. Their ECM is of two types, bone and cartilage, differing in their composition according to their physical and chemical function. The axial and appendicular skeleton is formed by endochondral ossification, where mesenchymal stem cells condence and differentiate into chondrocytes forming cartilage. The chondrocytes proliferate and terminally differentiate into hypertrophic cells and the cartilage is subsequently replaced by bone tissue.[66, 67] During this process the cartilage matrix, rich in collagen type II and X, is replaced by bone matrix predominantly consisting of collagen type I. This process also occurs in older individuals, in the growth plate, which is responsible for skeletal growth and the increase in bone length during childhood. Bone can also form without the cartilage template, in a process called intramembranous ossification. This occurs mainly in proximal sites, like the mandible and craniofacial skeleton, where condensing mesenchymal cells differentiate directly into bone. (Figure 1.6) These events are controlled by hormones and growth factors, as well as physical properties like cellular proximity and mechanical cues.[68] In this thesis, special interest is focused on the bone inducing growth factor BMP-2 and its role in bone and cartilage formation.

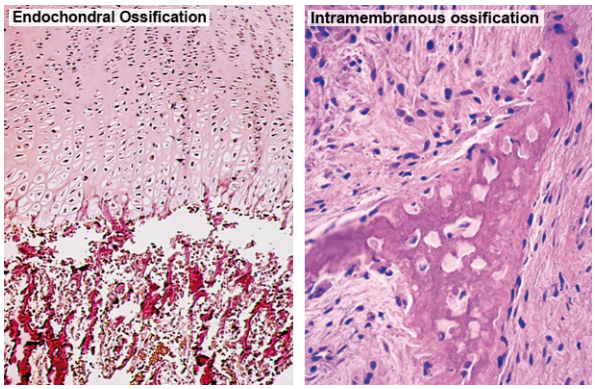

Figure 1.6 Endochondral bone formation starts with a cartilage template, where chondrocytes differentiate terminally into hypertrophic cells and subsequently die by apoptosis. The cartilage is mineralized and vascularized and the cartilage matrix is replaced by bone ECM. In intramembranous bone formation, bone is formed directly from condensing mesenchymal cell, without the cartilage template. The two types have different histological appearance.

BMP-2

BMPs are a group of more than 20 proteins being part of the transforming growth factor-β (TGF-β) family.[69] They are essential mediators in cell differentiation and play a central role in patterning formation in the early stage embryo and in the formation of skeletal elements during development.[70, 71] They were first discovered and purified from demineralized bone matrix and were characterized by their ability to induce bone formation at ectopic sites.[72]

During endochondral development, cartilage and bone differentiation involve a series of events that are directly influenced by BMPs.[73] BMP-2 is the most widely used growth factor to promote osteogenic differentiation, and it has also been shown to induce cartilage formation.[74, 75] In cartilage tissue, BMP-2 is expressed in the growth plate and regulates growth plate chondrogenesis by inducing chondrocyte proliferation and hypertrophy.[66, 67] Soluble BMP-2 protein acts in autocrine and paracrine fashions by binding extracellularly to transmembrane receptors and trigger an intracellular signaling cascade mediated by SMAD proteins.[76-78] (Figure 1.7)

In tissue engineering recombinant human BMP-2 has been used for several indications, including skull defects (calvaria), long bone (femur) and jaw bone (mandible).[79] The induction or acceleration of bone growth by delivery of BMP-2 strongly depends on the delivery method, but may also be affected by the source of BMP-2. Although recombinant human (rh) BMP-2 successfully has regenerated bone tissue, the success relies on high dosages compared to tissue levels.[79] To decrease the dosage there are two factors that can

be influenced: the carrier and the BMP-2 itself. In paper III, the osteoinductivity of BMP-2 produced in different cell lines with the rationale to produce more potent BMP-2 was investigated and the effects of its antagonist noggin.

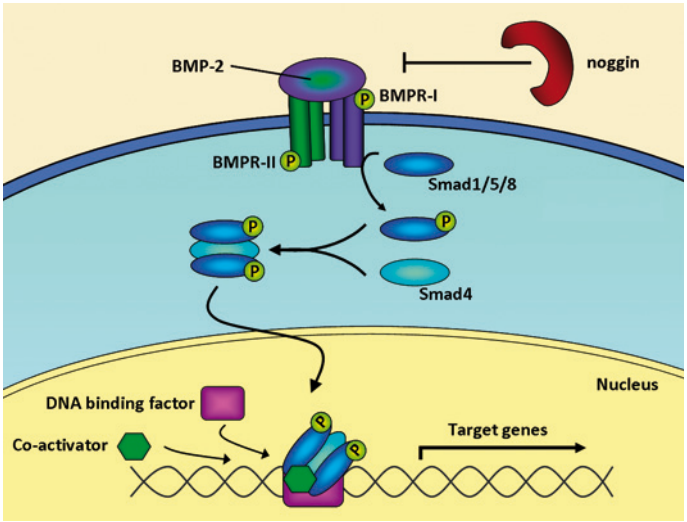

Figure 1.7 BMP-2 signaling is initiated by binding of BMP-2 to the BMP-receptors I and II. Upon binding the receptors are phosphorylated and a signaling pathway is started mediated by Smads, which are translocated into the nucleus and alter the expression of target genes. Noggin and other BMP antagonists bind with BMP-2, 4 and 7 and block BMP signalling. Modified from Chen et al. 2004.[9]

BMP-2 is also known to be involved in growth and differentiation of mesenchymal cells to chondrocytes during joint formation as well as play an important role in maintenance of articular cartilage in the adult.[80, 81] BMP-2 has been shown to differentiate mesenchymal cells into the chondrogenic lineage and to enhance the production of articular cartilage matrix *in vitro*[82, 83]. BMP-2 has also been implanted *in vivo* to heal cartilage defects[84]. Hence, it is an interesting candidate for the repair of cartilage defects. In paper IV, a hyaluronan hydrogel system was investigated as delivery vehicle of BMP-2 by chondrogenic differentiation of cells *in vitro* and cartilage regeneration *in vivo*.

Noggin – a BMP-2 regulatory factor

The regulation of BMP activity has been studied for many years and several BMP inhibitors have been identified, including noggin[85], chordin[86] and follistatin[87]. These are secreted factors that inactivate extracellular BMP-2 by direct binding. Noggin is required for neural tube formation, somite patterning and chondrogenesis.[88] Noggin deficient mice display short limbs and

overgrowth of cartilage due to excessive BMP signaling. However, they show a normal craniofacial skeleton, which is directly formed from condensing mesenchymal cells (intramembranous ossification), and a severely affected distal skeleton, which is cartilage-dependent upon formation.[89, 90] Noggin and osteoinductive BMPs, such as BMP-2 and BMP-4, are both expressed in chondrocytes during endochondral skeletal formation.[91] It has also been shown by *in vitro* experiments that BMP-2 induces the expression of noggin in osteoblasts.[92] The induction by BMP-2 of its own antagonist may limit the osteoinductive effects of BMP-2 in bone tissue.

Cartilage regeneration

Articular cartilage is a highly specialized tissue found at the ends of bones in articulating joints. It is an avascular supporting connective tissue, critical for resisting compression and reducing friction during joint movement (Figure 1.8). Articular cartilage functions in part due to cartilage specific components such as collagen type II, aggrecan and proteoglycans, specifically organized in a dense ECM.[93, 94] Cartilage has a low metabolic rate, making the regeneration and repair ability of the tissue limited. Age related degeneration, trauma and developmental disorders can result in pain and disability, which are problems affecting people of all ages. The ECM composition and organization of articular cartilage is maintained by the resident cells, chondrocytes, which make up less than 5% of the tissue in adult animals.[95]

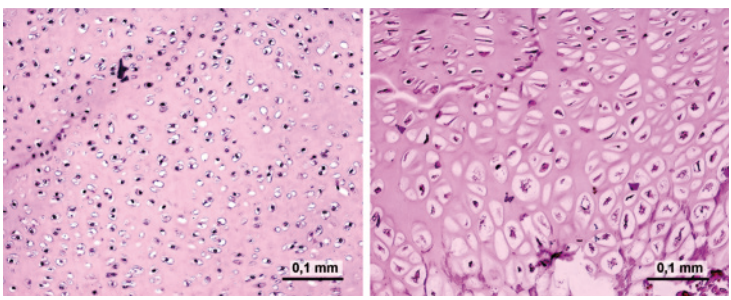

Figure 1.8 In growing long bone, cartilage is divided into two zones. At the joint surface is articular cartilage with small rounded cells, which persists throughout life (left). Near the mineralization zone, the chondrocytes have diffentiated into large hypertrophic cells, which subsequently die by apoptosis and the cartilage matrix is replaced by bone (right).

When cartilage damage occurs, some inherent regeneration may take place. However, the newly formed tissue is often fibrous cartilage, having inferiour mechanical properties compared to healthy articular cartilage.[96, 97] Many techniques, mostly surgical, are employed, but there is still no procedure that effectively provides long-term repair existing today. These include prosthetic joint replacement[98], surgical procedures[99] and tissue engineering strategies[42].

Although prosthetic joint replacement is successful, this invasive approach does not always provide long-term joint functionality due to loosening or limited life span of the prostheses.[100] Alternative treatment methods based on tissue engineering and regenerative medicine approaches usually include extraction and expansion of cells followed by seeding on a scaffold prior implantation. These have the aim of providing a long term solution with living functional tissue. Here, we used delivery of growth factors that would stimulate differentiation of endogenous cells to deposit ECM and regenerate the tissue.

2. Results and discussion

2.1 Collagen deposition on polymer scaffolds

In paper I and II, *in situ* deposition of ECM by human dermal fibroblasts onto knitted polymer meshes to create hybrid materials was investigated. Fibroblasts are cells that naturally produce and secrete ECM, in particular collagen type I. Different polymers and culture conditions were compared and structure and morphology of the composite material as well as the amount of collagen deposited by the seeded cells were evaluated.

In paper I, we investigated if a pulsed-flow bioreactor system creating dynamic culture conditions would provide efficient ECM deposition from normal fibroblast cells. The bioreactor system was developed from Tissue Works Ltd (Manchester, UK) where dynamic culture conditions were applied by a pulsatile, radial expansion of the scaffolds in the system set-up seen in figure 2.1.

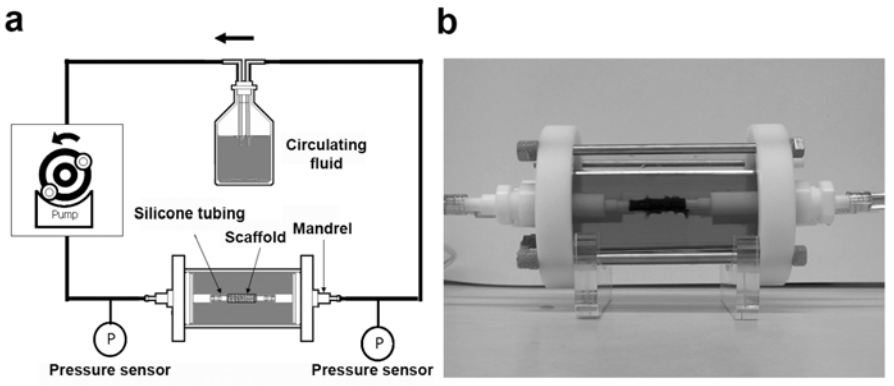

Figure 2.1 Set-up of the bioreactor system. (a) The peristaltic pump creates a pulsating flow that that stretches the material placed inside the reactor. (b) A picture showing the bioreactor with knitting placed around the silicone tubing attached to the mandrels inside the reactor chamber.

Cells were cultured on a compliant non-degradable poly(ethylene-terephtalate) (PET) mesh. PET was chosen as a support material to avoid polymer degradation leading to any possible changes in mechanical properties of the support material. Comparison of cells cultured under static and dynamic conditions were investigated, and we found that ECM deposition was favored by dynamic culture conditions, but the collagen deposition portion was low compared to total amount synthesized protein.

Cell proliferation and morphology

Cell proliferation on static and dynamic PET meshes was assayed at time points 0, 2, 4, 7 and 14 days by colorimetric Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Sigma-Aldrich). The MTT assay showed that the fibroblasts proliferated well on the meshes over a culture period of 2 weeks, but in dynamic culture they seemed to arrest in growth during the second week, indicating the cells had proliferated to cover the available growth surface (Figure 2.2).

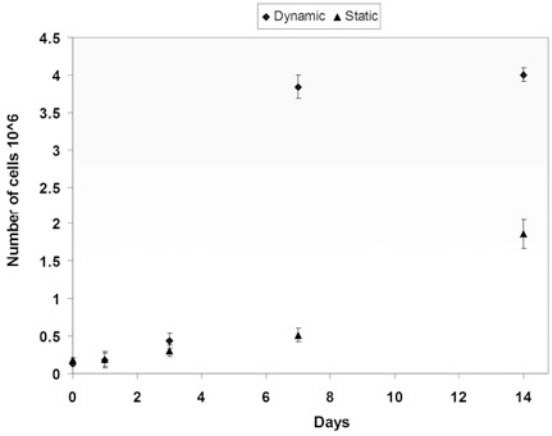

Figure 2.2 Plot of mean cell number over time for dynamic and static cultures. Dynamic culture conditions favor the proliferation of the fibroblasts in the PET meshes. Error bars represent the standard deviation of the mean of triplicate samples.

Scanning electron microscopy (SEM) and histology was used to visualize the deposition of ECM on the polymer meshes. Dynamic culture conditions led to faster proliferation and after two weeks in culture a layer of cells and ECM proteins was covering the surface of PET meshes (Figure 2.3a). At higher magnification the ECM film was seen to contain a fine fibrillar network, consistent with a high proportion of collagen (Figure 2.3b). Dynamic conditions also induced local orientation in the culture, with some cell and matrix fiber alignment noticed along the polymer fibers that was not ob-

served in static cultures (Figure 2.3c and d). Some preferential orientation in the direction of the fibers in the polymer support is expected, since fibroblasts tend to align and orientate themselves and the ECM when cultured in micro grooved substrates and on fiber structures, but this alignment would be present in both static and dynamic culturing.[101, 102] At a macroscopic level, however, the matrix seemed to be deposited in random orientation, not following strain directions for static or dynamic conditions. Since synthetic polymers are processable into a variety of shapes and sizes, the tendency of cells and ECM to locally align under mechanical conditions would provide a potential tool to affect the shape and structure of the biological component of the resulting composite material.

In knitted PGA meshes, fibroblasts and traces of matrix were seen in patches attached across fibers of each bundle (Figure 2.3e). Adherent fibroblasts utilized individual fibers but tended to form sheets around the bundles, more like a two dimensional surface (Figure 2.3f).

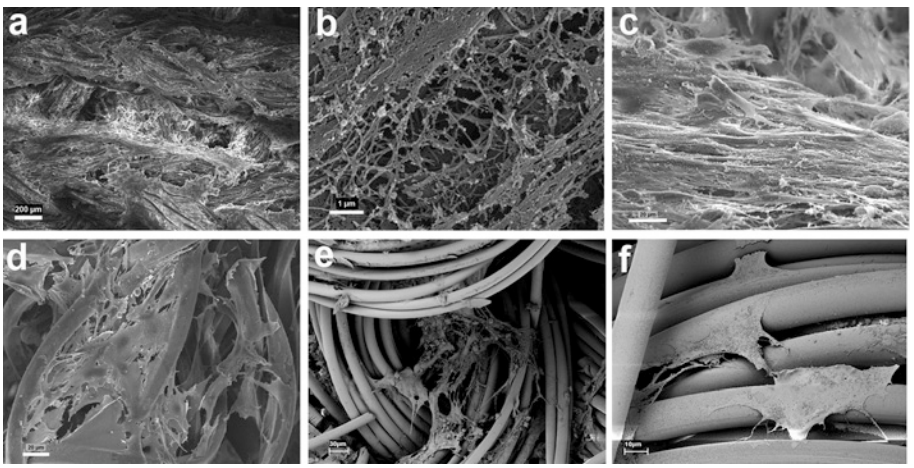

Figure 2.3 Human dermal fibroblasts grown for 14 days on PET mesh (a-d), showing the formation of a protein film over the polymer surface. The orientated fiber mesh structure in (a) is largely covered by a sheet of ECM in 14 days. Higher power of the ECM layer (b) shows the collagen fibril structure within the protein film. In dynamic culture conditions (c) alignment of the cells and deposited ECM along the polymer fiber direction is seen, while static cultures (d) show a more random distribution of cells with much less deposited ECM. There is little sign of matrix deposition on PGA after 14 days (e-f). Fiber bundles supporting cell attachment over and between polymer fibers with cell–cell attachment (e) and cell attachment to polymer fibers (f) is seen.

Cross section histology of two weeks samples showed a homogenous morphology of the constructs, indicating growth into a three dimensional structure. The porous structure provided efficient diffusion and transport of oxygen and nutrients in and out of the material giving the possibility for cells to

penetrate and migrate into the structure (Figure 2.4). Immunohistochemistry revealed the presence of fibronectin, laminin and elastin, mainly in the outer and inner side of the polymer support, indicating that a complex ECM assembly could be achieved *in vitro*.

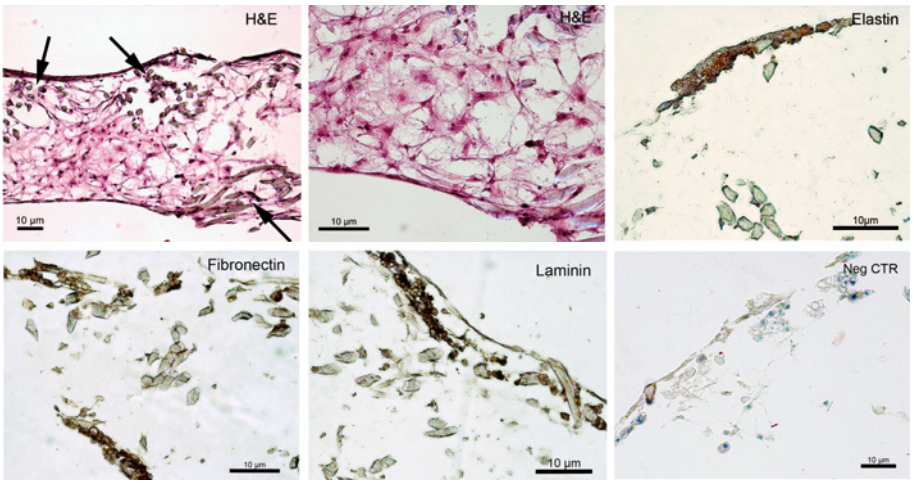

Figure 2.4 Hematoxylin and eosin stained histology sections of fibroblasts cultured on PET meshes showed a homogeneous cell distribution and morphology inside the scaffolds after 2 weeks in dynamic culture, with a more dense population of cells near the outer surface. Polymer fibers are marked with arrows. Immunohistochemistry showed the presence of laminin, fibronectin and elastin, mainly in the surface regions of the constructs.

Collagen deposition

In paper I, we could see that cells deposited a complex ECM, of which the main part was collagen. The amount of produced collagen was quantified using an HPLC method measuring the amount hydroxyproline (hyp) in the samples. Hyp is a rather unusual aminoacid, but which is common in collagen, where about 14% of all amino acids are hyp.[103] The results from paper I showed that collagen deposition was increased 5 fold under mechanical loading. However, the main part of the synthesized collagen was detected as soluble proteins in the culture medium, representing unprocessed and degraded collagen. (Table 2.1) In absolute terms, the total collagen accumulated in bioreactor culture was limited (microgram levels) and the rate of accumulation slowed down with increasing culture time. However, enough ECM is deposited to support the cells in three dimensions, which would be the purpose of the materials, but it is not comparable to the amounts found in native target tissues (20–30% collagen, e.g. skin and tendons).

Table 2.1 Collagen deposition on PET mesh in static and dynamic culture and degraded collagen present in the medium.

Culture period (weeks)	Deposited on mesh (µg/cm^2)	Total in medium (µg)	Proportion of deposited collagen (%)
Static			
2	12.1	194	6
4	10.8	331	3
6	18.4	758	2
Dynamic			
2	23.7	56	30
4	60.5	331	15
6	81.6	586	12

The rate limiting step

As seen in paper I and reported previously, tissue engineered constructs have modest collagen contents, even after long periods in culture.[104-107] The problems associated with low deposition concern the length and sophistication of the culture system, and our present limited ability to control the ECM produced in terms of structure or function. To tackle this systematically, we compared the deposition of collagen from different cells, under different culture conditions on two polymer supports, PGA (degradable polyester) and PET (non degradable polyester) meshes as discussed in paper II.

What we saw here, was that the use of three dimensional scaffolds, like the PGA and PET meshes, increased total collagen *synthesis* under static conditions by more than five-fold compared with two dimensional cultures. Total collagen synthesis was further increased 3.6-fold by the dynamic culture for PGA meshes and 5.4-fold for PET meshes. However, most of this collagen was degraded to ethanol-soluble peptides. (Table 2.2)

Even if all the collagen would be recovered from the medium it would not be functional for the fabrication of tissue-engineered constructs. It represents a rapid overall collagen turnover rather than useful *deposition*. Importantly, there was no increase in *deposited* (useful) collagen synthesis when moving from two dimensional well plate culture to three dimensional static mesh and a modest increase in collagen deposition was observed in pulsed mesh culture in comparison to collagen deposited by cells in two dimensional culture.

The concept of collagen deposition being dependent on tropocollagen (monomer) retention around the cell, i.e. by surrounding cells/ECM in the three dimensional configuration, is supported by the findings here and elsewhere.[108] To overcome the problem, we may need to address the problem from another angle, shifting from trying to increase synthesis to focus on increasing deposition rates. For fibroblasts and other mature matrix cells the matrix collagen deposition is greatest *between* cell layers rather than onto a polymer surface, and suggests that an ideal ECM culture system requires multi-layer fibroblasts in order to trap or deposit the maximal proportion of

the synthesized collagen. Notably, this configuration closely resembles that found *in vivo*.

Table 2.2. Collagen produced by neonatal dermal fibroblasts in culture

	Total collagen (µg)	**Deposited collagen (µg)**	**Deposition (%)**
Static PET mesh (11 days)	505±11	8±1.2	1.5
Static PGA mesh (11 days)	506±43	0	0
Dynamic PET bioreactor (5 days)	1268±123	14.8±12	1.1
Dynamic PGA bioreactor ($1x10^6$ cells, 5 days)	825±371	1.6±2.8	0.2
hDF adult (11 days)	118±3.3	11.2±10	10
hDF neonatal (11 days)	81±1.3	14±13	19

The central proposal arising from this study is that one effective strategy to successfully increase the proportion of new collagen incorporated into the ECM is to sterically retain newly synthesized collagen monomers close to the cell using nano-porous, as opposed to micro-porous, scaffolds. Based on these findings, it is suggested that next generation scaffolds are designed with cells pre-embedded in nano-fibrous meshes, such as collagen or fibrin gels. This strategy would deal with the key problem of low deposition by steric retention of collagen monomers from the start of the culture period. Such scaffolds may be made as hybrids of polymer meshes embedded in cellular collagen gels, as described elsewhere.[109-111]

2.2 BMP-2 expression and regulation

In Paper III and IV, another approach was applied where we wanted to affect endogenous cells to produce their own ECM *in vivo*, by adding growth factors to the system as compared to mechanical cues in Paper I and II. In our lab, we have previously worked with bone regeneration induced by BMP-2. Paper III is a pre-clinical study on the effects of cells over-expressing BMP-2, and the interplay between BMP-2 and its antagonist noggin. By understanding some of the underlying mechanisms, one would be able to produce more potent growth factors and potentially mimic an *in vivo* situation where expression of growth factors is closely related to the expression of their specific antagonists.[112] This may lead to a more optimized healing process.

Over-expression of BMP-2 in chondrocytes compared to MSC

In Paper III, secretion and processing of BMP-2 over-expression from virally transduced cells were compared. Chondrocytes, representing endochondral ossification and mesenchymal stem cells (MSC) representing intramembraneous ossification were examined, since they are influenced by BMP-2 and noggin signaling in different ways.

The osteogenic induction of media from BMP-2 expressing chondrocytes and MSC was analysed by alkaline phosphatase (ALP) assay, a marker for-osteoblast differentiation. The results showed that chondrocytes gave rise to higher osteoinduction compared to MSC (Figure 2.5a). Levels of secreted BMP-2 from the medium and intracellular BMP-2 from the cell layer were further analysed by western blot. Chondrocytes secreted fully mature, dimeric BMP-2 into the medium, which could be seen as a clear band at 32 kDa in western blots (Figure 2.5b). From MSC, almost all BMP-2 was trapped inside the cells as unprocessed precursors in sizes ranging from 110 kDa and lower and almost no BMP-2 could be detected from the medium (Figure 2.5c).

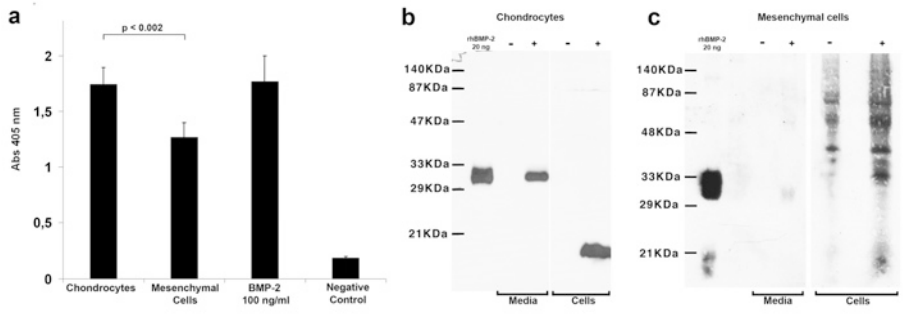

Figure 2.5 (a) The osteoinduction, as shown by ALP assay of collected media from transduced cells was higher in chondrocytes compared to mesenchymal cells. (b) Western blots of conditioned media and cell layer showed that chondrocytes secreted fully mature BMP-2 into the media, while (c) BMP-2 proteins from mesenchymal cells were detected from 110kDa and lower, suggesting that BMP-2 is unprocessed in these cells.

The secretion and hence osteoinduction may be influenced by furin, a protease that cleave pro-BMP-2 into an active molecule, a process known to take place in the trans-golgi network.[113] Immunocytochemistry staining for furin showed that in chondrocytes furin was detected within secretory vesicles in the cytoplasm, whereas in MSC, furin showed a perinuclear staining consistent with localization in the endoplasmatic reticulum (ER)/golgi network (Figure 2.6). The difference in localization indicates that processing and secretion of mature BMP-2 from chondrocytes is more efficient than in W20-17 cells. Previous studies have shown that the secretion of BMP-2 is

impaired in cells with low levels of furin, leaving the main fraction of BMP-2 as precursor proteins intracellulary.[114]

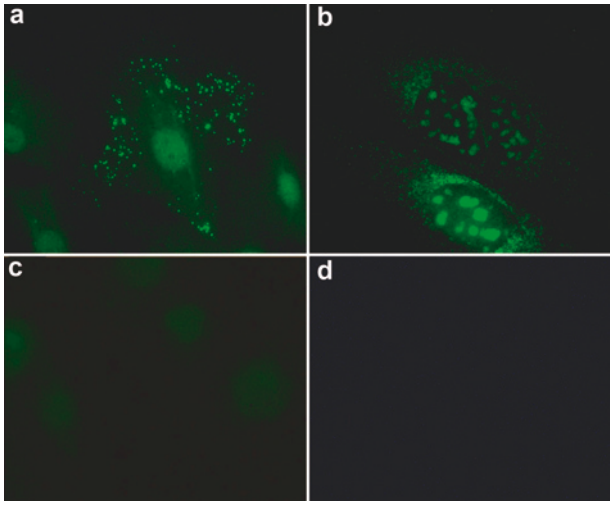

Figure 2.6 The distribution of furin in chondrocytes (a) and mesenchymal cells (b) were visualized by immunocytochemistry. In chondrocytes furin was detected within secretory vesicles in the cytoplasm, whereas in mesenchymal cells furin displayed a perinuclear staining pattern consistent with localization in the golgi/ER. Negative controls (c) and (d) were treated similarly except for omission of primary antibody.

The effect of noggin

The action of BMP-2 on cells is highly regulated by inhibitors, such as noggin. Noggin and osteogenic BMPs have been found to be co-expressed in chondrocytes during endochondral ossification.[91] To assess the distribution of BMP-2 and noggin in growing long bone we performed immunohistochemistry from human fetal femur (Figure 2.7). We found that BMP-2 was distributed in the entire cartilage ECM in metaphyses, whereas noggin was found in the pericellular zone surrounding chondrocytes, but not in the chondroid matrix. The strongest signal was found in the mineralization zone. Noggin was also detected in adjacent soft tissue including perichondrium. BMP-2 and noggin are both expressed in chondrocytes, but occupy different zones in the ECM, whereas BMP-2 and noggin exhibit an overlapping pattern in osteoprogenitors. The co-localization of BMP-2 and noggin in the mineralization zone, a site where bone formation is occurring was surprising; since noggin would inhibit the action of BMP-2 hence hinder the formation of bone. We believe that noggin localized in soft tissues adjacent to cartilage inhibits the effect of BMP-2, while noggin in the mineralization zone modulates the action of BMP-2. To investigate the interacting effects of noggin and BMP-2 expression in mesenchymal cells, we co-infected W20-17 cells for over-expression of both factors.

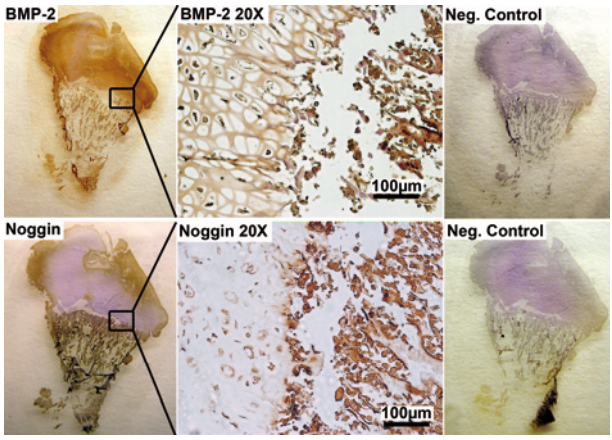

Figure 2.7 Immunohistochemical staining of a fetal human femur, showing presence of BMP-2 in the extracellular matrix in the entire cartilage area, and of noggin in the pericellular matrix surrounding chondrocytes (left). An overlapping extracellular staining pattern for noggin and BMP-2 was found in the mineralization zone in the epiphysis (middle).

Unexpectedly, the amount of secreted BMP-2 was greatly enhanced in these cells as compared to cells expressing BMP-2 alone (Figure 2.8a). A potential mechanism is that noggin complexes with BMP-2 and promotes release from a cell bound fraction. Since BMP-2 contains a heparin binding site the majority of processed BMP-2 is probably immobilized and bound to membrane-associated heparan sulphates.[115] The increased amount of BMP-2 in the conditioned media can alternatively be explained by protection of noggin-BMP-2 complex from degradation increasing its half-life. Noggin-bound BMP-2 may consequently accumulate in the conditioned culture medium, whereas non-bound BMP-2 rapidly degrades. The secreted BMP-2 in co-expressing cells was functional in vitro and in vivo with a 5-fold increase in ALP-activity and the induction of abundant ectopic bone respectively (Figure 2.8b).

In complementary experiments, a construct (nogORF) that gave greatly enhanced yields of noggin was used to transduce the cells. With this construct, the amounts secreted BMP-2 from MSC was greatly enhanced compared to nog3' used previously (Figure 2.9a and b). However, the bone induction *in vivo* was dependent on expression level of noggin since high yields of noggin completely inhibited osteoinduction, despite enhanced levels of BMP-2 secretion detected in western blot (Figure 2.8c). Thus, the ratio of noggin to BMP-2 seem to be important to obtain a stabilizing or modulating effect of noggin on bone formation, which is in accordance with a previous study utilizing transplanted transduced mesenchymal cells over-expressing noggin and BMP-4 for cranial repair.[116]

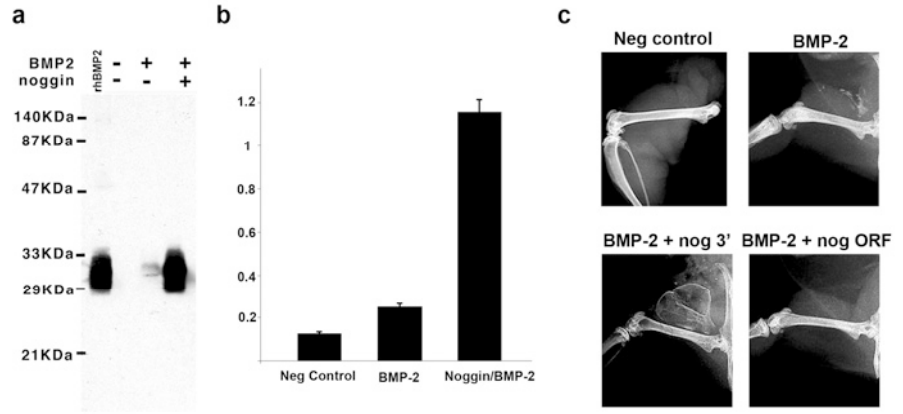

Figure 2.8 (a) Co-expression of noggin and BMP-2 in mesenchymal cells dramatically enhanced the secretion and processing of BMP-2 compared to cells expressing BMP-2 and control vector, as shown by western blot. (b) Mesenchymal cells over-expressing both noggin and BMP-2 (noggin/BMP-2) displayed approximately 5 times higher ALP activity than cells over expressing BMP-2 alone. (c) Induction of ectopic bone formation *in vivo* showed that co-infected (BMP-2 + nog 3') cells showed more ectopic bone than BMP-2 alone (BMP-2) However, this effect is dependent on noggin expression level since high expression from nogORF and BMP-2 completely inhibited the osteoinduction.

However, in nogORF transduced chondrocytes the cellular fraction of BMP-2 was completely depleted, without increasing the detected levels in the medium (Figure 2.9c). A possible explanation is that since free BMP-2 in conditioned media was collected by heparin sepharose beads the non-heparin binding fraction of BMP-2 did not bind to the beads. Noggin-bound BMP-2 may conceal the heparin-binding sites on both proteins and the complex becomes non heparin binding. Alternatively, the noggin-BMP-2 complex involves a heparan sulphate, which occupies the heparin-binding sites.

The present results raise the possibility that the BMP-2-noggin complex can dissociate extracellularly, releasing an active BMP-2 molecule. The difference in BMP-2 levels from co-expressing MSCs and chondrocytes implies that the noggin-BMP-2 complex, or noggin-heparan sulphate-BMP-2 complex, can dissociate in MSC and consequently deposit a larger fraction of free BMP-2. A cell-specific factor, such as a heparan sulphatase, that is selectively expressed in MSC but not in chondrocytes affecting the stability of the noggin-BMP-2 complex must exist to support this hypothesis and needs to be further investigated.[117, 118]

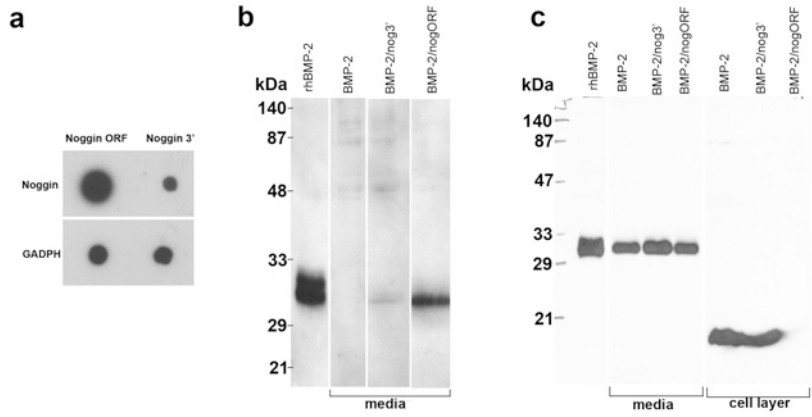

Figure 2.9 (a) Dot blot showing that the noggin ORF construct gives higher noggin yields compared to the noggin-3'UTR construct. Levels were compared using GADPH as an internal standard. (b) Western blot, showing that noggin-3'UTR and BMP-2 co-expression in mesenchymal cells causes enhanced level of BMP-2 secreted into the media, whereas high noggin expression (nogORF) induced even higher level of BMP-2. Cells over-expressing BMP-2 alone secreted a non-detectable level at this exposure time. (c) In chondrocytes the co-expression of noggin and BMP-2 did not affect the amounts of BMP-2 secreted into the media collected by heparin-sepharose beads. In the cell layer high noggin expression completely depleted BMP-2. Note, part of this blot is also presented in figure 2.5.

2.3 Cartilage regeneration

In paper IV, a hyaluronan hydrogel system developed in our lab and previously used for bone repair was investigated for use in cartilage regeneration together with the bone and cartilage promoting growth factor BMP-2. The gel properties when used together with cells were investigated *in vitro* and the possibility of healing cartilage defects in a pilot study in rabbits was investigated *in vivo*.

Gel properties and cartilage differentiation *in vitro*

To assess the possibility of the hyaluronan hydrogel system to retain cells and induce chondrogenesis, an *in vitro* study was performed where differentiation of chondrocytes and mesenchymal cells cultured in the gel was examined by histology and immunohistochemistry. GAGs, which support the ECM proteins in the biomechanical function of cartilage, were assessed by the colorimetric 1,9-dimethyl-methylene blue (DMMB) assay. The cells were cultured *in vitro* for 28 days in chondrogenic medium consisting of Dulbecco's modified eagles medium-Ham's F12 (DMEM-F12) supplemented with insulin-transferring-sodium selenite (ITS), and ascorbic acid.

Mesenchymal cells were further supplemented with 100ng/ml BMP-2. The samples were compared to pellet culture, which is a common procedure for differentiating chondrocytes and mesenchymal cells *in vitro*[119-121], and collagen gel, a commonly used biomaterial.[122]
Cells were encapsulated in the hyaluronan gel system using a dual barrel syringe with a mixing tip (Figure 2.10a). Live/dead staining with ethidium homodimer (red color, dead cells) and calcein (green color, live cells) showed that 96% of the cells were viable after gel setting (Figure 2.10b). Contraction of scaffolds, like collagen based materials[122, 123] is a well known problem in tissue engineering, depending on cellular expression of alfa-smooth muscle actin, exerting contractile forces on the material. This is similar to the contraction taking place during wound healing.[124] The hyaluronan hydrogel kept its original shape throughout the culture period (Figure 2.10). The ability of the hyaluronan gel to maintain its shape implies that it could be adapted or molded when injected directly into a defect or body cavity and stay stable until degraded. Some degradation of the gel was observed, which was seen as as a decrease in stiffness and mechanical stability of the gel. This could be due to degradation by hyaluronidases, which chondrocytes and mesenchymal cells are known to express during chondrogenesis.[125]

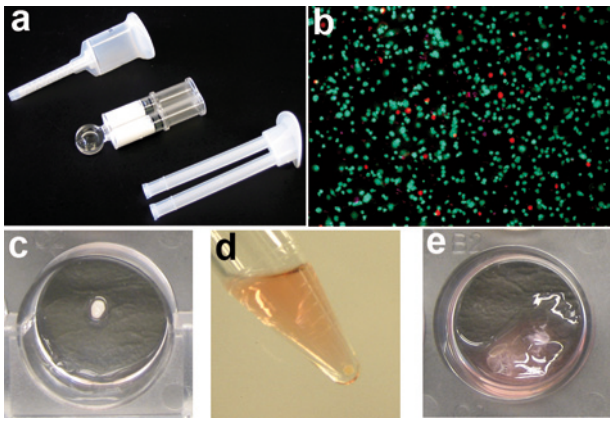

Figure 2.10 (a) The gels were prepared using a dual barrel syringe with mixing tip. (b) live/dead staining, where viable cells are green and dead cells red, showing that chondrocytes had high viability immediately after gel setting. Collagen type I scaffolds (c) used for cartilage differentiation were highly contracted after 4 days of culture, reduced almost to the size of pellets (b) while hyaluronan gels (e) kept their size throughout the culture period.

Cartilage tissue is rich in ECM, and at the end of the culture period histological and immunohistochemical analysis revealed a cartilage-like tissue with cells embedded in lacunae in an extensive ECM. The ECM stained positive for collagen type II and aggrecan, two markers for cartilage (Figure 2.11a). The ECM was rich in GAGs as could be seen from the metachro-

matic staining from toluidine blue. The GAG content was also quantified using the DMMB assay, showing that in hyaluronan gels the GAG content, normalized to number of cells, was comparable to the amount in pellet culture (Figure 2.11b). However, the pellet culture is limited to approximately 200 000 cells before the mass is to dense to allow diffusion of nutrients and waste leading to cell death in the core of the mass.[126] The total amount of produced GAG per sample (as opposed to GAG per cell) was higher for both chondrocytes and MSC, 38 µg/ml and 24 µg/ml compared to 10 µg/ml, for pellet culture, indicating that larger constructs of tissue engineered cartilage could be achieved by using the hyaluronan gel system. In the hyaluronan hydrogel system the size of the constructs could be increased five-fold in terms of volume and number of cells, as shown in the *in vitro* study.

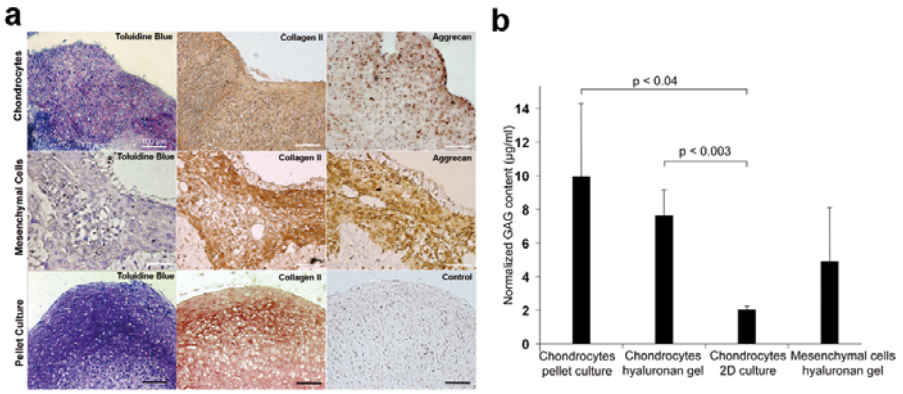

Figure 2.11 (a) Histology and immunohistochemistry revealed a tissue engineered cartilage-like structure with cells embedded in an extensive ECM, consisting of collagen type II and aggrecan. The metachromatic staining from toluidine blue revealed a GAG rich matrix. (b) The GAG content in samples was quantified using the DMMB assay, where chondrocytes and mesenchymal cells (MSC) deposited GAG amounts comparable to pellet culture.

Cartilage regeneration *in vivo* by delivery of BMP-2

For *in vivo* use, cells and/or growth factors can be suspended in one of the components prior to injection and a solid, crosslinked gel is formed *in situ* upon mixing. The hyaluronan gel system has previously been used as a growth factor carrier for bone regeneration in rat and pig models involving recruitment of endogenous cells.[43, 127] A non-cell based strategy leaves out the tedious work of expanding and delivering cells to the damaged site, but requires the presence of intrinsic stem or progenitor cells. In cartilage the only potential source of progenitor cells is from the underlying bone marrow in the subchondral bone.[128] We performed a study on femoral osteochondral defects in rabbit, where the material containing the cartilage and bone pro-

moting growth factor BMP-2 was injected into the joint cavity and was able to affect the entire joint surface, rather than filling the defect. This model could be expanded for use on osteoarthritis models. A defect of 4 mm diameter was created on both femoral condyles in the knee joint of 6 month old New Zealand White rabbits. The same defects were created on the contralateral knees, but the defect was left untreated to serve as a control. In total were 12 knees operated on. The defects were created by careful drilling down to subchondral bone. After assuring access to stem cells from subchondral bone by bleeding, the patella was relocated in position and the knee capsule tightly closed. A volume of 0.5 ml of the gel containing 150 µg/ml BMP-2 (n=3) or the hydrogel system alone (n=3), was injected into the knee joints, with a dual barrel syringe with mixing tip before the skin wound was closed (Figure 2.12).

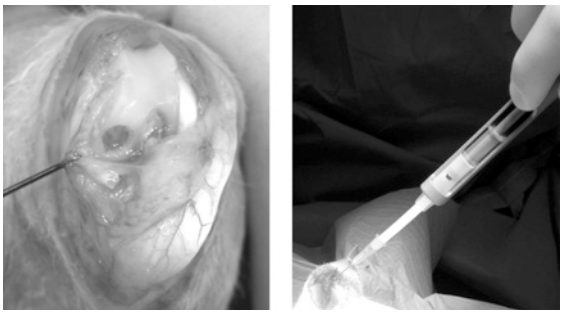

Figure 2.12 Cartilage defects were created on both femoral condyles by drilling. Subsequently, the knee capsule was closed and the material injected into the joint cavity.

Three months post-operatively the animals were sacrificed and the osteochondral defects underwent gross morphology and histopathological examinations. No visual signs of inflammation could be seen in the knees, showing that the gel is biocompatible without any side effects when placed in the joint area. However, during the first week after operation, the rabbits favored the operated control leg over the one containing the materials to some extent, indicating some discomfort from the placement of the hydrogel. After the first week, no lameness was observed and they use both legs without difference. The discomfort in gel treated knees could originate from an intraarticular pressure created by the presence of material, which diminished as the gel started to degrade and the pressure disappeared. After this initial post-operative period the animals were in a good general condition, as seen by their steady weight gain throughout the experimental period.

The gross morphology examinations of the joint surface showed that the defects, regardless of treatment group, were filled with whitish regenerated tissue, which was distinguishable from surrounding tissue. The defects in medial condyles were generally better filled compared to lateral condyles,

which could be due to an anatomically rabbit-specific tendon that partly covers the lateral condyle and may affect regeneration.[129] No distinguishable difference in gross morphology between the groups was observed. Trichrome stained histology cross sections from the centre of the defect area were analysed according to the modified scoring system from Sellers et al. where scoring ranges from 0 (normal cartilage) to 31 points (no repair tissue).[130] The total scores from two investigators blinded to the treatment groups and their average values are reported in figure 2.13.

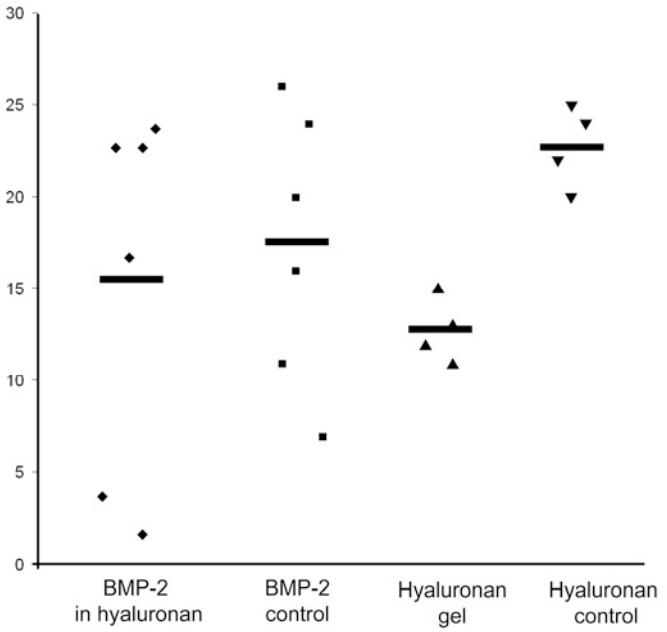

Figure 2.13 Histological scoring of regenerated tissue according to Sellers et al., where a low scoring value represents high degree of regeneration. The total score from each joint, determined by two observers are reported as points in the plot. In total, 12 knees were operated, were contralateral knees on each rabbit were left untreated to serve as control for their respective treatment. The arithmetic mean of treatment groups is shown as horizontal bars. ♦ BMP-2 in hyaluronan gel (n=3); ■ operated control to BMP-2 treated knees (n=3); ▲ hyaluronan gel treated (n=2, one animal was sacrificed prematurely due to infection in one knee joint); ▼ operated control to hyaluronan treated knees (n=2)

Due to large variations within the BMP-2 in hyaluronan group and their respective control operated knees, no difference between the groups was seen here, 15.5±10 versus 17.3±7.4. The hyaluronan gel treated groups, however, showed lower total score 12.8±1.7 compared to control operated, 22.8±2.2. Earlier studies in rabbits and humans have shown that injections of hyalu-

ronan (native) may have a healing effect on articular cartilage by placing it intraarticularly.[131-133] Immunohistochemistry revealed that the regenerated tissue was of better quality in both the treated groups as can be seen by the more intense staining of collagen type II (articular cartilage) compared to collagen type I (fibrocartilage). In addition, the subchondral bone in these two groups showed a more compact appearance in the histology sections compared to untreated control knees (Figure 2.14). The results from this pilot study are encouraging, and to obtain statistical verification a complete animal study should be conducted with larger number of animals in each group. The hyaluronan hydrogel system has shown very promising results, and could be used both for cell based and cell free regeneration therapies.

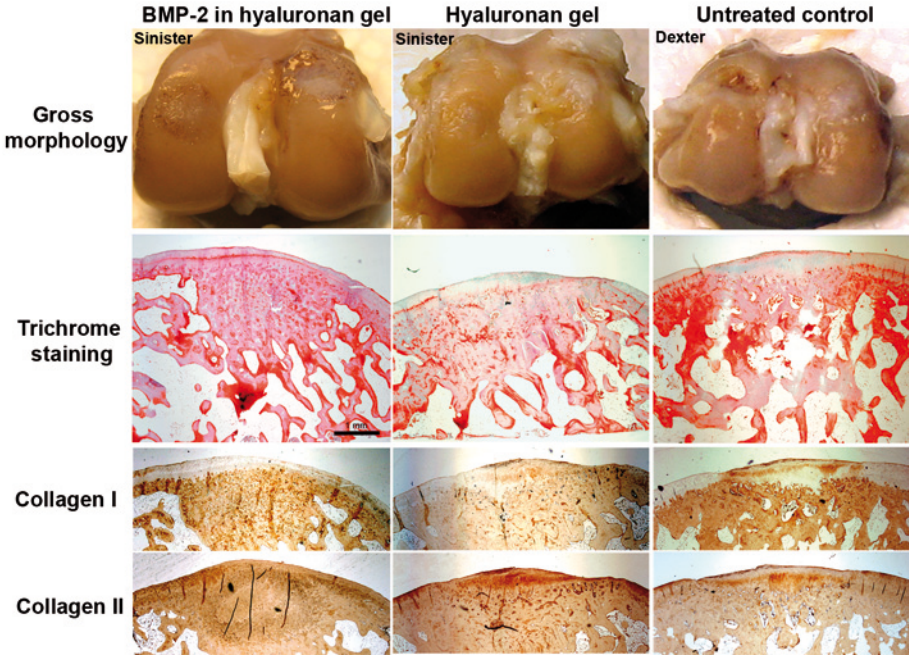

Figure 2.14 Evaluation of the cartilage regeneration by gross morphology, histology and immunohistochemistry of samples from knee joints treated with BMP-2 in hyaluronan gel, hyaluronan gel and untreated controls. From gross morphology and trichrome staining it was difficult to distinguish any differences in healing. However, when staining for collagen type I (fibro cartilage) and collagen type II (articular cartilage) a clear difference between treated an non treated group could be seen, where BMP-2 treated defects showed a collagen type II rich regenerated tissue, while untreated controls had a larger element of collagen type I in the defect area. Scale bar is 1 mm.

3. Concluding remarks and future perspectives

ECM, growth factors and cells all interact to define the cellular microenvironment. These interactions play a crucial role in regulating cellular physiology both *in vivo* and *in vitro*. The aim of the present work has been to design ECM based materials by influencing cells cultured *in vitro* and regenerating ECM rich tissues *in vivo*.

In paper I, a method for the *in vitro* production of ECM–polymer hybrid scaffolds by dynamic culture of cells in a pulsed-flow bioreactor system was investigated. These results demonstrated the principle of producing biomimetic polymer–ECM composites, while not supporting the use of bioreactors for the production of gram-level collagen found in tissues. The system could be extended for potential development of more sophisticated materials, where these composite materials could be designed to contain specific biological information through usage of genetically modified cells, enriching the ECM-polymer hybrid with growth factors or other morphogens. It is also possible to incorporate cell guidance cues into the system through synthetic fiber surface topology.

In paper II, we tested the rate-limiting processes of collagen accumulation in a number of representative two and three dimensional bioreactor configurations used in tissue engineering. This quantitative analysis showed that collagen synthesis rates, although low, can be increased to some extent by culture conditions. However, the real rate limiting factor was the low proportion of this new collagen which is deposited to functional ECM. There is strong circumstantial evidence that this may improve after prolonged culture, when ECM does accumulate to the level that keeps the new collagen from diffusing away. Based on these findings, it is suggested that next generation scaffolds could be designed with cells pre-embedded in nano-fibrous meshes, such as collagen or fibrin gels. This approach would deal with retention of collagen monomers from the start of the culture period.

In paper III, we investigated the BMP-2 processing and secretion in two cell systems representing endochondral (chondrocytes) and intramembranous (MSC) bone formation. Our results revealed that chondrocytes are cells designed to deliver BMP-2 and induce mineralization by resident or recruited MSC. The MSC population itself is not as efficient in BMP processing, but may be manipulated by low overexpression of the BMP antagonist noggin to greatly enhance its capacity to deliver BMP-2. We believe that noggin may have a modulating effect on BMP-2, where noggin complexes

with BMP-2 and protect it from degradation. The results suggest that the binding of noggin and BMP-2 is reversible and that a third, possibly cell specific, factor could influence the complex stability. The results may also be relevant in determining the appropriate cell type for the production of recombinant BMP-2 since one would benefit from the natural processing mechanisms present in chondrocytes or in noggin-BMP-2 co-expressing MSC. There are still many unanswered questions that need to be addressed and further investigations of the interplay between BMP-2, noggin and a possible third factor are interesting to pursue to find a more efficient way to produce and deliver BMP-2 for bone regeneration.

In paper IV, an injectable hyaluronan hydrogel system was evaluated for differentiating mesenchymal stem cells and chondrocytes *in vitro*. The material was also tested in a pilot study in rabbits to heal articular cartilage defects *in vivo*. *In vitro* tissue engineered constructs from the gel system showed typical cartilage morphology with cells embedded in lacunae in an extensive extracellular matrix, consisting of collagen type II and aggrecan. For the *in vivo* experiments, a non cell-based regeneration method was applied where cartilage regeneration by delivery of BMP-2 from the gel was examined. The defects healed well, but the difference between treated and control groups were hard to distinguish as assessed by histology. However, the quality of the regenerated tissue was better in the treated groups with more collagen type II (articular cartilage) compared to collagen type I (fibrocartilage). Furthermore, the results indicated that the hyaluronan gel alone had a beneficial effect on cartilage regeneration. The hyaluronan hydrogel system showed promising results as a versatile biomaterial for cartilage regeneration, which could easily be placed intraarticularily and can be used for both cell based and cell free therapies. The system could be expanded to osteoarthritis models and investigations concerning administration of other drugs from the hyaluronan hydrogel or hyaluronan gels functionalized with cartilage promoting substances are interesting lines of research to pursue in the quest of finding functional treatments for patients.

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5. Svensk sammanfattning

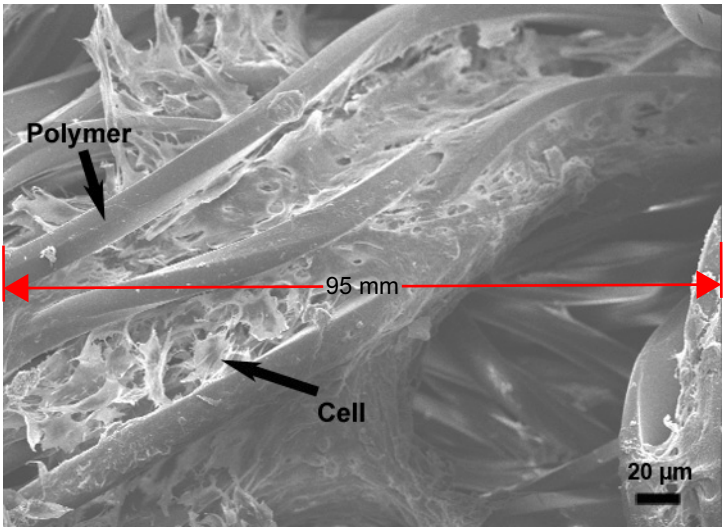
Vävnadsregenerering är ett forskningsområde där nya material utvecklas som kan ersätta skadade vävnader och organ samtidigt som det ska fungera praktiskt i en operationssal. Genom att utnyttja syntetiska material tillsammans med levande celler och olika vävnadsinducerande substanser kan man påverka kroppen att återskapa ny vävnad. Celler producerar och utsöndrar normalt sett sin egen omgivning i form av ett nätverk av naturliga stora molekyler och proteiner som kallas extracellulärmatris (ECM). Nybildning och ombildning av vävnad sker under hela vår livstid, i allt från fosterutveckling till sårläkning. Dessa förändringar är förknippade med delning av celler, liksom utsöndring och omstrukturering av ECM. ECM är huvudbeståndsdelen i bindväv såsom hud, ben och brosk och står för struktur och mekaniska egenskaper. Den spelar också en viktig roll för utveckling och underhåll av kroppens vävnad och dess funktioner.

Målet med detta arbete har varit att utveckla ECM baserade material genom att påverka celler som odlas *in vitro* (utanför kroppen), samt regenerera ECM rika vävnader *in vivo* (i kroppen).

De syntetiska materialen, eller bärarna, inom vävnadsregenerering skall fungera som konstgjord ECM, en tillfällig byggnadsställning för kroppens egna celler under läkning och nybildning av vävnad. ECM är uppbyggd av stora strukturella molekyler, där sammansättningen ger vävnaden dess specifika egenskaper och utseende. Det är svårt att syntetisk kunna tillverka material med samma tredimensionella struktur och komplexa innehåll som naturlig ECM.

I den första studien i denna avhandling har vi undersökt möjligheten att tillverka ECM genom att låta celler växa på ett nät av syntetisk polymer och därigenom utsöndra ECM som fastnar på nätet. På det här sättet får vi ett hybridmaterial, med en syntetisk del (polymernätet) och en biologisk del (ECM) där man kan dra fördel från båda delarnas egenskaper (Figur 5.1). Cellerna odlades under dynamiska förhållanden i en bioreaktor och vi kunde detektera en komplex ECM av flera olika beståndsdelar, även om utbytet var relativt lågt. Eftersom utbytet var lågt, undersökte vi i en annan studie det hastighetsbegränsande steget vid ECM produktion. Vi gjorde en kvantitativ analys av utsöndring (total mängd producerad ECM) och deposition (funktionell ECM som fastnar på polymernätet) av det strukturella proteinet kollagen, som utgör huvudbeståndsdelen i ECM. Denna analys visade att den största begränsningen var den låga andelen kollagen som *deponeras som*

funktionell ECM. Större delen av det cellerna producerar försvinner ut i odlingsmediet utan att det sätts ihop i ett nätverk. Emellertid såg vi att när nätverk av kollagen väl bildats hindrade detta den nybildade kollagenet att försvinna iväg, och större andel ingick i ny ECM på polymernätet. För att effektivisera bildandet av funktionell ECM föreslår vi att man i framtiden tillverkar dessa material av t ex en syntetisk komponent (polymernätet) och celler inbäddade i en gel av t ex kollagen framrenat från djur som därvid hindrar nyproducerat kollagen att försvinna och ökar andelen funktionell, deponerad matris och minskar odlingstiden.



Figur 5.1 Svepelektronmikroskopi-bild av celler som växer på polymernätets fibrer. Cellerna utsöndrar ECM som brygger mellan olika fibrer och till slut täcks hela nätet av ECM och celler.

Vi har i en annan studie undersökt möjligheten att påverka kroppens egna celler att producera ECM *in vivo*. Detta kan man göra genom att implantera tillväxtfaktorer i ett bärarmaterial som påverkar kroppens egna stamceller att bilda ny vävnad. Tillväxtfaktorer är alltså en sorts vävnadsinducerande substanser. Här arbetade vi med ben och brosk, vilket är vävnader som innehåller en mycket hög andel ECM. För att inducera dess två vävnader kan man använda tillväxtfaktorn bone morphogenetic protein-2 (BMP-2).

BMP-2 kan tillverkas till exempel genom att sätta in BMP-2-genen i en värdcell som sedan utsöndrar BMP-2. Vi undersökte mekanismerna bakom BMP-2 utsöndring från celler och styrning av dess aktivitet i kroppen. Det visade sig att broskceller är mer effektiva att producera BMP-2 jämfört med benmärgsstamceller, samt att en negativ regulator till BMP-2, noggin, spelar en mycket viktig roll för att styra var i kroppen BMP-2 skall vara aktiv och inducera benbildning och var den inte skall göra det.

BMP-2 är också viktig för att bilda och underhålla broskvävnad. Broskskador och förslitningar är idag ett stort problem för många människor. I en studie undersökte vi möjligheten att behandla broskskador genom att frisätta BMP-2 i knäleden från en injicerbar gel baserad på den modifierade ECM komponenten hyaluronsyra. Vi kunde visa att broskceller och benmärgsstamceller kan bilda broskvävnad i gelen *in vitro* samt att den hade viss läkande effekt *in vivo*. Studien visar uppmuntrande resultat, men det finns många frågor kvar som måste bevaras innan man i framtiden skulle kunna behandla patienter på detta sätt.

6. References

1. Langer, R, and Vacanti, JP (**1993**). Tissue Engineering. *Science* 260: 920-926.
2. Bergman, K (**2008**). Hyaluronan Derivatives and Injectable Gels for Tissue Engineering. p. 51. Universitetsbiblioteket, Uppsala.
3. van Blitterswijk, C, Thomsen, P, Lindahl, A*, et al.* (**2008**). *Tissue homeostasis* In: Tissue Engineering, Academic Press.
4. Adams, JC, and Watt, FM (**1993**). Regulation of development and differentiation by the extracellular matrix. *Development* 117: 1183-1198.
5. Martin, P (**1997**). Wound healing - Aiming for perfect skin regeneration. *Science* 276: 75-81.
6. Ingber, DE, and Levin, M (**2007**). What lies at the interface of regenerative medicine and developmental biology? *Development* 134: 2541-2547.
7. Carmeliet, P (**2000**). Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395.
8. Cohen, S, and Elliott, GA (**1963**). The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. *Journal of Investigative Dermatology* 40: 1-5.
9. Chen, D, Zhao, M, and Mundy, GR (**2004**). Bone morphogenetic proteins. *Growth Factors* 22: 233-241.
10. Kleinman, HK, Philp, D, and Hoffman, MP (**2003**). Role of the extracellular matrix in morphogenesis. *Curr Opin Biotechnol* 14: 526-532.
11. Breitkreutz, D, Mirancea, N, and Nischt, R (**2009**). Basement membranes in skin: unique matrix structures with diverse functions? *Histochemistry and Cell Biology* 132: 1-10.
12. Huber, MM, Trattnig, SM, and Lintner, FM (**2000**). Anatomy, Biochemistry, and Physiology of Articular Cartilage. *Investigative Radiology* 35: 573-580.
13. Kuure, S, Vuolteenaho, R, and Vainio, S (**2000**). Kidney morphogenesis: cellular and molecular regulation. *Mechanisms of Development* 92: 31-45.
14. Ottani, V, Martini, D, Franchi, M, Ruggeri, A, and Raspanti, M (**2002**). Hierarchical structures in fibrillar collagens. *Micron* 33: 587-596.
15. van der Rest, M, and Garrone, R (**1991**). Collagen family of proteins. *Faseb Journal* 5: 2814-2823.

16. Raines, EW (**2000**). The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. *International Journal of Experimental Pathology* 81: 173-182.
17. Ratcliffe, A (**2000**). Tissue engineering of vascular grafts. *Matrix Biology* 19: 353-357.
18. Laurent, GJ, Chambers, RC, Hill, MR, and McAnulty, RJ (**2007**). Regulation of matrix turnover: fibroblasts, forces, factors and fibrosis. *Biochemical Society Transactions* 035: 647-651.
19. Sasisekharan, R, Raman, R, and Prabhakar, V (**2006**). Glycomics approach to structure-function relationships of glycosaminoglycans. *Annu Rev Biomed Eng* 8: 181-231.
20. Hwang, NS, Varghese, S, Lee, HJ*, et al.* (**2007**). Response of zonal chondrocytes to extracellular matrix-hydrogels. *FEBS Letters* 581: 4172-4178.
21. Tognana, E, Borrione, A, De Luca, C, and Pavesio, A (**2007**). Hyalograft (R) C: Hyaluronan-based scaffolds in tissue-engineered cartilage. *Cells Tissues Organs* 186: 97-103.
22. Varghese, S, Hwang, NS, Canver, AC, Theprungsirikul, P, Lin, DW, and Elisseeff, J (**2008**). Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biology* 27: 12-21.
23. White, E, Baralle, F, and Muro, A (**2008**). New insights into form and function of fibronectin splice variants. *The Journal of Pathology* 216: 1-14.
24. Hynes, RO (**1990**). *Fibronectins*, Springer-Verlag: New York.
25. Kubota, Y, Kleinman, H, Martin, G, and Lawley, T (**1988**). Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* 107: 1589-1598.
26. Tzu, J, and Marinkovich, MP (**2008**). Bridging structure with function: Structural, regulatory, and developmental role of laminins. *The International Journal of Biochemistry & Cell Biology* 40: 199-214.
27. Humphries, SM, Lu, Y, Canty, EG, and Kadler, KE (**2008**). Active Negative Control of Collagen Fibrillogenesis in Vivo. *Journal of Biological Chemistry* 283: 12129-12135.
28. Kanagawa, M, Michele, DE, Satz, JS*, et al.* (**2005**). Disruption of perlecan binding and matrix assembly by post-translational or genetic disruption of dystroglycan function. *FEBS Letters* 579: 4792-4796.
29. Ruggiero, F, and Koch, M (**2008**). Making recombinant extracellular matrix proteins. *Methods* 45: 75-85.
30. Yang, SF, Leong, KF, Du, ZH, and Chua, CK (**2001**). The design of scaffolds for use in tissue engineering. Part 1. Traditional factors. *Tissue Engineering* 7: 679-689.
31. Ratner, BD, Hoffmann, AS, Schoen, FJ, and Lemons, JE (**2004**). *Biomaterials Science*, Elsevier Academic Press: San Diego.

32. Levenberg, S, Langer, R, and Gerald, PS (**2004**). Advances in tissue engineering. *Current Topics in Developmental Biology* 61: 113-134.
33. Sokolsky-Papkov, M, Agashi, K, Olaye, A, Shakesheff, K, and Domb, AJ (**2007**). Polymer carriers for drug delivery in tissue engineering. *Advanced Drug Delivery Reviews* 59: 187-206.
34. Harley, BA, Freyman, TM, Wong, MQ, and Gibsony, LJ (**2007**). A new technique for calculating individual dermal fibroblast contractile forces generated within collagen-GAG scaffolds. *Biophys J* 93: 2911-2922.
35. Stenzel, KH, Miyata, T, and Rubin, AL (**1974**). Collagen as a biomaterial. *Annual Review of Biophysics and Bioengineering* 3: 231-253.
36. Faraj, KA, Van Kuppevelt, TH, and Daamen, WF (**2007**). Construction of collagen scaffolds that mimic the three- dimensional architecture of specific tissues. *Tissue Engineering* 13: 2387-2394.
37. Inoue, Y, Anthony, JP, Lleon, P, and Young, DM (**1996**). Acellular human dermal matrix as a small vessel substitute. *Journal of Reconstructive Microsurgery* 12: 307-311.
38. Bannasch, H, Unterberg, T, Fohn, M, Weyand, B, Horch, RE, and Stark, GB (**2008**). Cultured keratinocytes in fibrin with decellularised dermis close porcine full-thickness wounds in a single step. *Burns* 34: 1015-1021.
39. Kleinman, HK, and Martin, GR (**2005**). Matrigel: Basement membrane matrix with biological activity. *Seminars in Cancer Biology* 15: 378-386.
40. Badylak, SF, Tullius, R, Kokini, K*, et al.* (**1995**). The use of xenogenic small-intestinal submucosa as a biomaterial for achilles-tendon repair in a dog-model. *Journal of Biomedical Materials Research* 29: 977-985.
41. Hubbell, JA (**2003**). Materials as morphogenetic guides in tissue engineering. *Curr Opin Biotechnol* 14: 551-558.
42. Temenoff, JS, and Mikos, AG (**2000**). Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* 21: 431-440.
43. Bergman, K, Engstrand, T, Hilborn, J, Ossipov, D, Piskounova, S, and Bowden, T (**2009**). Injectable cell-free template for bone-tissue formation. *Journal of Biomedical Materials Research Part A* 91A: 1111-1118.
44. Balazs, EA (**2008**). Hyaluronan as an ophthalmic viscoelastic device. *Curr Pharm Biotechnol* 9: 236-238.
45. Kirker, KR, Luo, Y, Nielson, JH, Shelby, J, and Prestwich, GD (**2002**). Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing. *Biomaterials* 23: 3661-3671.
46. Laurent, TC, Gelotte, B, and Hellsing, K (**1964**). Cross-linked gels of hyaluronic acid. *Acta Chemica Scandinavica* 18: 274-275.
47. Gao, JM, Niklason, L, and Langer, R (**1998**). Surface hydrolysis of poly(glycolic acid) meshes increases the seeding density of vascular

smooth muscle cells. *Journal of Biomedical Materials Research* 42: 417-424.
48. Cima, LG, Ingber, DE, Vacanti, JP, and Langer, R (**1991**). Hepatocyte culture on biodegradable polymeric substrates. *Biotechnology and Bioengineering* 38: 145-158.
49. Hubbell, JA (**1998**). Synthetic biodegradable polymers for tissue engineering and drug delivery. *Current Opinion in Solid State & Materials Science* 3: 246-251.
50. Bryant, SJ, Davis-Arehart, KA, Luo, N, Shoemaker, RK, Arthur, JA, and Anseth, KS (**2004**). Synthesis and characterization of photopolymerized multifunctional hydrogels: Water-soluble poly(vinyl alcohol) and chondroitin sulfate macromers for chondrocyte encapsulation. *Macromolecules* 37: 6726-6733.
51. Grayson, WL, Ma, T, and Bunnell, B (**2004**). Human Mesenchymal Stem Cells Tissue Development in 3D PET Matrices. *Biotechnology Progress* 20: 905-912.
52. Engelberg, I, and Kohn, J (**1991**). Physicomechanical properties of degradable polymers used in medical applications - a comparative study *Biomaterials* 12: 292-304.
53. Miller, RA, Brady, JM, and Cutright, DE (**1977**). Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. *Journal of Biomedical Materials Research* 11: 711-719.
54. Brown, RA, Prajapati, R, McGrouther, DA, Yannas, IV, and Eastwood, M (**1998**). Tensional homeostasis in dermal fibroblasts: Mechanical responses to mechanical loading in three-dimensional substrates. *Journal of Cellular Physiology* 175: 323-332.
55. Kim, BS, Nikolovski, J, Bonadio, J, and Mooney, DJ (**1999**). Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. *Nature Biotechnology* 17: 979-983.
56. Lee, CH, Shin, HJ, Cho, IH*, et al.* (**2005**). Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast. *Biomaterials* 26: 1261-1270.
57. O'Callaghan, CJ, and Williams, B (**2000**). Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells - Role of TGF-beta(1). *Hypertension* 36: 319-324.
58. Suresh, S (**2006**). Biomechanics and biophysics of cancer cells. In *European Conference on Computational Fluid Dynamics*, pp. 3989-4014. Pergamon-Elsevier Science Ltd, Egmond, NETHERLANDS.
59. Eastwood, M, Mudera, VC, McGrouther, DA, and Brown, RA (**1998**). Effect of precise mechanical loading on fibroblast populated collagen lattices: Morphological changes. *Cell Motility and the Cytoskeleton* 40: 13-21.
60. Kisiday, JD, Frisbie, DD, McIlwraith, CW, and Grodzinsky, AJ (**2009**). Dynamic Compression Stimulates Proteoglycan Synthesis by Mesenchymal Stem Cells in the Absence of Chondrogenic Cytokines. *Tissue Engineering Part A* 15: 2817-2824.

61. McCulloch, AD, Harris, AB, Sarraf, CE, and Eastwood, M (**2004**). New multi-cue bioreactor for tissue engineering of tubular cardiovascular samples under physiological conditions. *Tissue Engineering* 10: 565-573.
62. Bilodeau, K, and Mantovani, D (**2006**). Bioreactors for tissue engineering: Focus on mechanical constraints. A comparative review. *Tissue Engineering* 12: 2367-2383.
63. El Haj, AJ, Hampson, K, and Gogniat, G (**2009**). Bioreactors for Connective Tissue Engineering: Design and Monitoring Innovations. *Bioreactor Systems for Tissue Engineering*, vol. 112. Springer-Verlag Berlin: Berlin. pp 81-93.
64. Martin, I, Wendt, D, and Heberer, M (**2004**). The role of bioreactors in tissue engineering. *Trends in Biotechnology* 22: 80-86.
65. Wendt, D, Riboldi, SA, Cioffi, M, and Martin, I (**2009**). Potential and Bottlenecks of Bioreactors in 3D Cell Culture and Tissue Manufacturing. *Adv Mater* 21: 3352-3367.
66. Davidson, ENB, Vitters, EL, van Lent, P, de Loo, F, van den Berg, WB, and van der Kraan, PM (**2007**). Elevated extracellular matrix production and degradation upon bone morphogenetic protein-2 (BMP-2) stimulation point toward a role for BMP-2 in cartilage repair and remodeling. *Arthritis Research & Therapy* 9.
67. De Luca, F, Barnes, KM, Uyeda, JA*, et al.* (**2001**). Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. *Endocrinology* 142: 430-436.
68. Karsenty, G, Kronenberg, HM, and Settembre, C (**2009**). Genetic Control of Bone Formation. *Annual Review of Cell and Developmental Biology* 25: 629-648.
69. Wu, X, Shi, W, and Cao, X (**2007**). Multiplicity of BMP signaling in skeletal development. *Annals of the New York Academy of Sciences* 1116: 29-49.
70. Hogan, BLM (**1996**). Bone morphogenetic proteins in development. *Current Opinion in Genetics & Development* 6: 432-438.
71. Zou, H, Choe, KM, Lu, Y, Massague, J, and Niswander, L (**1997**). BMP signaling and vertebrate limb development. In *Cold Spring Harbor Symposium on Quantitative Biology - Pattern Formation During Development*, pp. 269-272, Plainview, New York.
72. Urist, MR (**1965**). Bone - Formation by autoinduction. *Science* 150: 893-&.
73. Alden, TD, Pittman, DD, Hankins, GR*, et al.* (**1999**). In Vivo Endochondral Bone Formation Using a Bone Morphogenetic Protein 2 Adenoviral Vector. *Human Gene Therapy* 10: 2245-2253.
74. Shea, CM, Edgar, CM, Einhorn, TA, and Gerstenfeld, LC (**2003**). BMP treatment of C3H10T1/2 mesenchymal stem cells induces both chondrogenesis and osteogenesis. *Journal of Cellular Biochemistry* 90: 1112-1127.

75. Yoon, ST, and Boden, SD (**2002**). Osteoinductive molecules in orthopaedics: Basic science and preclinical studies. *Clin Orthop Rel Res*: 33-43.
76. Sakou, T, Onishi, T, Yamamoto, T, Nagamine, T, Sampath, KT, and Tendijke, P (**1999**). Localization of Smads, the TGF-beta 2 Family Intracellular Signaling Components During Endochondral Ossification. *Journal of Bone and Mineral Research* 14: 1145-1152.
77. Schmitt, JM, Hwang, K, Winn, SR, and Hollinger, JO (**1999**). Bone morphogenetic proteins: An update on basic biology and clinical relevance. *Journal of Orthopaedic Research* 17: 269-278.
78. Wu, CJ, and Lu, HK (**2008**). Smad signal pathway in BMP-2-induced osteogenesis - a mini review. *Journal of Dental Sciences* 3: 13-21.
79. Cowan, CM, Soo, C, Ting, K, and Wu, B (**2005**). Evolving concepts in bone tissue engineering. *Current Topics In Developmental Biology* 66: 239-285.
80. Rountree, RB, Schoor, M, Chen, H*, et al.* (**2004**). BMP receptor signaling is required for postnatal maintenance of articular cartilage. *PLoS Biol* 2: 1815-1827.
81. Lyons, K, Pelton, R, and Hogan, B (**1990**). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development* 109: 833-844.
82. Luyten, FP, Yu, YM, Yanagishita, M, Vukicevic, S, Hammonds, RG, and Reddi, AH (**1992**). Natural bovine osteogenin and recombinant human bone morphogenetic protein-2b are equipotent in the maintenance of proteoglycans in bovine articular-cartilage explant cultures. *Journal of Biological Chemistry* 267: 3691-3695.
83. Sailor, LZ, Hewick, RM, and Morris, EA (**1996**). Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. *J Orthop Res* 14: 937-945.
84. Sellers, RS, Zhang, RW, Glasson, SS*, et al.* (**2000**). Repair of articular cartilage defects one year after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). *Journal of Bone and Joint Surgery-American Volume* 82A: 151-160.
85. Smith, WC, and Harland, RM (**1992**). Expression cloning of noggin, a new dorsalizing factor localized to the spemann organizer in Xenopus embryos. *Cell* 70: 829-840.
86. Zhang, D, Ferguson, CM, O'Keefe, RJ, Puzas, JE, Rosier, RN, and Reynolds, PR (**2002**). A Role for the BMP Antagonist Chordin in Endochondral Ossification. *Journal of Bone and Mineral Research* 17: 293-300.
87. Reddi, AH (**2001**). Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. *Arthritis Research* 3: 1-5.
88. McMahon, JA, Takada, S, Zimmerman, LB, Fan, CM, Harland, RM, and McMahon, AP (**1998**). Noggin-mediated antagonism of BMP

signaling is required for growth and patterning of the neural tube and somite. *Genes & Development* 12: 1438-1452.
89. Brunet, LJ, McMahon, JA, McMahon, AP, and Harland, RM (**1998**). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280: 1455-1457.
90. Zimmerman, LB, DeJesusEscobar, JM, and Harland, RM (**1996**). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.
91. Pathi, S, Rutenberg, JB, Johnson, RL, and Vortkamp, A (**1999**). Interaction of Ihh and BMP/Noggin Signaling during Cartilage Differentiation. *Developmental Biology* 209: 239-253.
92. Gazzerro, E, Gangji, V, and Canalis, E (**1998**). Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *Journal of Clinical Investigation* 102: 2106-2114.
93. Cancedda, R, Dozin, B, Giannoni, P, and Quarto, R (**2003**). Tissue engineering and cell therapy of cartilage and bone. *Matrix Biology* 22: 81-91.
94. Watanabe, H, Yamada, Y, and Kimata, K (**1998**). Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. *Journal of Biochemistry* 124: 687-693.
95. Stockwel.Ra (**1967**). Cell density of human articular and costal cartilage. *Journal of Anatomy* 101: 753-&.
96. Cuevas, P, Burgos, J, and Baird, A (**1988**). Basic fibroblast growth-factor (FGF) promotes cartilage repair in vivo. *Biochem Biophys Res Commun* 156: 611-618.
97. Darling, EM, and Athanasiou, KA (**2003**). Biomechanical strategies for articular cartilage regeneration. *Ann Biomed Eng* 31: 1114-1124.
98. Ayers, DC, Dennis, DA, Johanson, NA, and Pellegrini, VD (**1997**). Common complications of total knee arthroplasty. *Journal of Bone and Joint Surgery-American Volume* 79A: 278-311.
99. Bouwmeester, P, Kuijer, R, Homminga, GN, Bulstra, SK, and Geesink, RGT (**2002**). A retrospective analysis of two independent prospective cartilage repair studies: autogenous perichondrial grafting versus subchondral drilling 10 years post-surgery. *J Orthop Res* 20: 267-273.
100. Dozin, B, Malpeli, M, Camardella, L, Cancedda, R, and Pietrangelo, A (**2002**). Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. *Matrix Biology* 21: 449-459.
101. Curtis, A, and Wilkinson, C (**1997**). Topographical control of cells. *Biomaterials* 18: 1573-1583.
102. Engelmayr, GC, Papworth, GD, Watkins, SC, Mayer, JE, and Sacks, MS (**2006**). Guidance of engineered tissue collagen orientation by large-scale scaffold microstructures. *Journal of Biomechanics* 39: 1819-1831.

103. Dalton, SJ, Mitchell, DC, Whiting, CV, and Tarlton, JF (**2005**). Abnormal extracellular matrix metabolism in chronically ischemic skin: A mechanism for dermal failure in leg ulcers. *Journal of Investigative Dermatology* 125: 373-379.
104. Ahsan, T, Chen, A, Chin, L, and al., e (**2003**). Effects of long-term growth on tissue engineered cartilage. *Trans Orthop Res Soc* 28:309.
105. Aulin, C, Foroughi, F, Brown, R, and Hilborn, J (**2009**). Extracellular matrix-polymer hybrid materials produced in a pulsed-flow bioreactor system. *Journal of Tissue Engineering and Regenerative Medicine* 3: 188-195.
106. Beekman, B, Verzijl, N, Bank, RA, von der Mark, K, and TeKoppele, JM (**1997**). Synthesis of collagen by bovine chondrocytes cultured in alginate; Posttranslational modifications and cell-matrix interaction. *Experimental Cell Research* 237: 135-141.
107. Cheema, U, Nazhat, SN, Alp, B*, et al.* (**2007**). Fabricating tissues: Analysis of farming versus engineering strategies. *Biotechnology and Bioprocess Engineering* 12: 9-14.
108. L'Heureux, N, Paquet, S, Labbe, R, Germain, L, and Auger, FA (**1998**). A completely biological tissue-engineered human blood vessel. *Faseb Journal* 12: 47-56.
109. Ananta, M, Aulin, CE, Hilborn, J*, et al.* (**2009**). A Poly(Lactic Acid-Co-Caprolactone)-Collagen Hybrid for Tissue Engineering Applications. *Tissue Engineering Part A* 15: 1667-1675.
110. Brown, RA, Wiseman, M, Chuo, CB, Cheema, U, and Nazhat, SN (**2005**). Ultrarapid engineering of biomimetic materials and tissues: Fabrication of nano- and microstructures by plastic compression. *Advanced Functional Materials* 15: 1762-1770.
111. Nazhat, SN, Abou Neel, EA, Kidane, A*, et al.* (**2007**). Controlled microchannelling in dense collagen scaffolds by soluble phosphate glass fibers. *Biomacromolecules* 8: 543-551.
112. Yoshimura, Y, Nomura, S, Kawasaki, S, Tsutsumimoto, T, Shimizu, T, and Takaoka, K (**2001**). Colocalization of noggin and bone morphogenetic protein-4 during fracture healing. *Journal of Bone and Mineral Research* 16: 876-884.
113. Zhou, A, Webb, G, Zhu, X, and Steiner, DF (**1999**). Proteolytic Processing in the Secretory Pathway. *Journal of Biological Chemistry* 274: 20745-20748.
114. Meleady, P, Henry, M, Gammell, P*, et al.* (**2008**). Proteomic profiling of CHO cells with enhanced rhBMP-2 productivity following co-expression of PACEsol. *PROTEOMICS* 8: 2611-2624.
115. Ruppert, R, Hoffmann, E, and Sebald, W (**1996**). Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *European Journal of Biochemistry* 237: 295-302.
116. Peng, H, Usas, A, Hannallah, D, Olshanski, A, Cooper, GM, and Huard, J (**2005**). Noggin Improves Bone Healing Elicited by Muscle Stem Cells Expressing Inducible BMP4. *Mol Ther* 12: 239-246.

117. Dhoot, GK, Gustafsson, MK, Ai, X, Sun, W, Standiford, DM, and Emerson, CP, Jr. (**2001**). Regulation of Wnt Signaling and Embryo Patterning by an Extracellular Sulfatase. *Science* 293: 1663-1666.
118. Selleck, SB (**2000**). Proteoglycans and pattern formation - Sugar biochemistry meets developmental genetics. *Trends in Genetics* 16: 206-212.
119. Holtzer, H, Abbott, J, Lash, J, and Holtzer, S (**1960**). The loss of phenotypic traits by differentiated cell in vitro. 1. Dedifferentiation of cartilage cells *Proceedings of the National Academy of Sciences of the United States of America* 46: 1533-1542.
120. Manning, WK, and Bonner, WM (**1967**). Isolation and culture of chondrocytes from human adult articular cartilage *Arthritis and Rheumatism* 10: 235-&.
121. Meyer, U, and Wiesmann, HP (**2006**). *Bone and cartilage engineering*, Springer-Verlag Heidelberg.
122. Noth, U, Rackwitz, L, Heymer, A*, et al.* (**2007**). Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels. *Journal of Biomedical Materials Research Part A* 83A: 626-635.
123. Yokoyama, A, Sekiya, I, Miyazaki, K, Ichinose, S, Hata, Y, and Muneta, T (**2005**). In vitro cartilage formation of composites of synovium-derived mesenchymal stem cells with collagen gel. *Cell and Tissue Research* 322: 289-298.
124. Qiu, W, Murray, MM, Shortkroff, S, Lee, CR, Martin, SD, and Spector, M (**2000**). Outgrowth of chondrocytes from human articular cartilage explants and expression of alpha-smooth muscle actin. *Wound Repair and Regeneration* 8: 383-391.
125. Bastow, ER, Byers, S, Golub, SB, Clarkin, CE, Pitsillides, AA, and Fosang, AJ (**2008**). Hyaluronan synthesis and degradation in cartilage and bone. *Cellular and Molecular Life Sciences* 65: 395-413.
126. Johnstone, B, Hering, TM, Caplan, AI, Goldberg, VM, and Yoo, JU (**1998**). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Experimental Cell Research* 238: 265-272.
127. Docherty-Skogh, A-C, Bergman, K, Jensen-Waern, M*, et al.* (**2009**). Bone morphogenetic protein-2 delivered by hyaluronan-based hydrogel induces massive bone formation and healing of cranial defects in minipigs. *Plastic and reconstructive surgery* In press.
128. Richter, W (**2009**). Mesenchymal stem cells and cartilage in situ regeneration. *Journal of Internal Medicine* 266: 390-405.
129. MacLaughlin, C, and Chiasson, RB (**1979**). *Laboratory anatomy of the rabbit*, McGraw-Hill Science/Engineering/Math.
130. Sellers, RS, Peluso, D, and Morris, EA (**1997**). The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. *Journal of Bone and Joint Surgery-American Volume* 79A: 1452-1463.

131. Kang, SW, Bada, LP, Kang, CS*, et al.* (**2008**). Articular cartilage regeneration with microfracture and hyaluronic acid. *Biotechnology Letters* 30: 435-439.
132. Karakok, M, Ugras, S, Tosun, N, Fuat, A, and Aydin, A (**2001**). Effect of intra-articular administration of hyaluronan and cortisone on secondary osteoarthritis of the infected rabbit's knee. *Tohoku Journal of Experimental Medicine* 195: 35-42.
133. Karlsson, J, Sjogren, LS, and Lohmander, LS (**2002**). Comparison of two hyaluronan drugs and placebo in patients with knee osteoarthritis. A controlled, randomized, double-blind, parallel-design multicentre study. *Rheumatology* 41: 1240-1248.

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