Tissue Engineering Strategies to Improve Cellular Repair: Harnessing the Regenerative Potential of Bone Marrow-Derived Stem and Progenitor Cells

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To Sarah-Trúclinh Tran, *my* marrow and my ride home

To my parents and theirs

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AC	articular chondrocyte
ACI	autologous chondrocyte implantation
acLDL	acetylated low density lipoprotein
Ad-MSC	adipose-derived mesenchymal stem cell
ANOVA	analysis of variance
APase	alkaline phosphatase
BaSMC	baboon smooth muscle cell
bFGF	basic fibroblast growth factor
BM-MSC	bone marrow-derived mesenchymal stem cell
BMP	bone morphogenetic protein
BSA	bovine serum albumin
COMP	cartilage oligomeric matrix protein
COX	cyclooxygenase
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ePTFE	expanded polytetrafluoroethylene
Fab	fragment antigen binding
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HIF	hypoxia-inducible factor
HRE	hypoxia responsive element
HSC	hematopoietic stem cell

HUVEC	human umbilical vein endothelial cell
IF	immunofluorescence
IgG	immunoglobulin G
KDR	kinase domain-containing receptor
mAb	monoclonal antibody
MACI	matrix-induced autologous chondrocyte implantation
MC	mesothelial cell
MMP	matrix metalloprotease
mRNA	messenger RNA
MSC	mesenchymal stem cell
MVEC	microvascular endothelial cell
N-cadherin	neural cadherin
N-CAM	neural cell adhesion molecule
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
OA	osteoarthritis
PBMNC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
Pe-MSC	periosteum-derived mesenchymal stem cell
PGI_2	prostacyclin
PI(3)K	phosphatidylinositol-3-OH kinase
P-selectin	platelet selectin
PTHrP	parathyroid hormone-related protein
qPCR	real-time quantitative polymerase chain reaction
RGD	arginine-glycine-aspartate
rhKDR	recombinant human KDR/Fc chimera
RNA	ribonucleic acid
RT	room temperature
Runx2/Cbfa1	runt-related transcription factor 2/core-binding factor α1
sGAG	sulfated glycosaminoglycan
SMC	smooth muscle cell

SM-MSC	synovial membrane-derived mesenchymal stem cell
SOX9	(sex determining region Y)-box 9
TBS	tris-buffered saline
TGF-β	transforming growth factor-β
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2

Abstract

Tissue Engineering Strategies to Improve Cellular Repair: Harnessing the Regenerative Potential of Bone Marrow-Derived Stem and Progenitor Cells

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Tissue engineering is a promising therapeutic option for injured tissues with deficient endogenous cellular repair such as atherosclerotic lesions of the vascular wall and chondral lesions of the synovial joints. Current cell-based surgical options use autologous tissue that may be of limited supply or the harvest of which can cause donor-site problems. As part of the body's natural repair process, immature bone marrow-derived cells may be recruited to sites of both diseased vascular wall and damaged articular cartilage. Both vascular engineering with circulating endothelial progenitor cells (EPCs) and cartilage engineering with mesenchymal stem cells (MSCs) are limited by the efficiency and rate of differentiation of these primitive precursors to functional cells resembling endothelial cells and articular chondrocytes. The goal of these studies was to develop tissue engineering platforms that are tailored for the use of bone marrow-derived stem and progenitor cells and that are compatible with existing vascular and chondral interventions. The different natures of these tissues present distinct challenges for cell-based regenerative strategies with regard to geometry, the compartmentalization of the relevant stem/progenitor cells, and environmental factors. For the tissues of interest here, the feasibilities of two different approaches were evaluated based on these challenges and the desire to shorten or eliminate expansion and differentiation times. The proposed strategy for vascular engineering is to avoid isolation altogether by capturing EPCs directly from flow to promote an endothelial monolayer on solid surfaces. In this work, the ability to do so with antibodies directed at the endothelial-specific receptor KDR was investigated and characterized *in vitro* with model cells. On the other hand, MSCs that are capable of chondrogenesis are physically separated from cartilage, residing in the hypoxic environment of the bone marrow. The proposed strategy for cartilage engineering is to transfer MSCs from bone marrow to cartilage defects following the *in vitro* formation of three-dimensional aggregates of differentiated MSCs. The goal of this work was to evaluate the effect of culturing MSCs under low oxygen during differentiation in microscopic aggregates designed to overcome mass transport limitations that can exist with culture of larger three-dimensional tissues. These studies answer critical questions about the feasibility of these tissue engineering strategies and lay the foundation for further development towards clinical relevancy.

CHAPTER I

Introduction

As biomedical engineers, we strive to use knowledge of biology, chemistry, and physics to design the devices and treatments to help ameliorate the health problems in our societies. The goal of this research was to develop tissue engineering platforms that are tailored for the use of bone marrow-derived stem and progenitor cells and that are compatible with existing medical technologies and intervention strategies. This work is divided into two parts – related to two distinct injuries – although both focused on the limitations of current cell-based therapies of the relevant ailments. Diseases and damage afflicting both vascular walls and articular cartilage are among the most common causes of pain, disability, and death in the United States. Due to the high prevalence and the associated burden of injuries to these specialized tissues, there have been multiple treatments explored for both and with increasing knowledge and technological capabilities, tissue engineering strategies have escalated. Still, robust engineered replacement tissues are lacking for both.

Bone marrow is a reservoir of stem and progenitor cells that may be dispatched as part of the healing response to injuries of numerous of the body's tissues. Immature bone marrow-derived cells may be recruited to sites of both diseased vascular wall and damaged articular cartilage as part of the body's natural repair process. Therefore, in serious cases where medical interventions are required, the relevant bone marrow-derived cell populations are considered promising candidates for administered cellular therapies. However, such treatments are still limited due to a number of factors – not least among them a relatively poor understanding of the characteristics that define appropriate cells and of the signals that drive the uncommitted cells down desired lineages. With the seemingly exponential increases in knowledge being unearthed by experimental biologists, realistic treatments that harness the powers of bone marrow-

derived cells for repair of these tissues may not be far off. It will be one job of biomedical engineers to develop the platforms that can exploit these advances for therapeutic gains.

The research described here was devised with an interest in injuries that require intervention and with an eye towards methods that lend themselves to integration with current biomedical strategies. Atherosclerotic lesions and chondral lesions are debilitating injuries with deficient endogenous cellular repair, making them prime candidates for tissue engineering interventions. Vascular grafts and autologous chondrocyte implantation (ACI) were long ago developed options but both have yet to evolve to incarnations capable of stimulating suitable long-term tissue replacement. They are both promising intervention strategies that would benefit greatly from modifications to improve long-term viability and integration with the native tissue. The common principle of the two platforms proposed and studied here is that they were conceived of with autologous bone marrow-derived stem/progenitor cells in mind. These cells have a great regenerative potential and represent a cell source more easily and painlessly isolated than adult vascular endothelial cells (ECs) and articular chondrocytes (ACs). The described platforms were designed with an awareness of one of the persistent shortcomings of progenitor and stem cells, namely the long culture periods required for differentiation to the desired cell type. For immunogenic reasons the patients own cells are attractive of course; however, stem and progenitor cells tend to suffer from lengthy expansion and/or lengthy differentiation procedures. To address this challenge for the tissues of interest here, two different approaches were taken that were deemed appropriate to the relevant tissues and existing intervention technologies discussed: a method designed to avoid isolation and expansion altogether by stimulating endothelial progenitor cell (EPC) recruitment to solid surfaces *in vivo* and a method designed to make more efficient the *in vitro* chondrogenic differentiation of mesenchymal stem cells (MSCs).

As the bone marrow-derived progenitor cells capable of endothelial differentiation circulate in peripheral blood, the strategy evaluated for vascular intervention was the capture of flowing cells onto solid surfaces via an important endothelial growth factor receptor expressed by these cells. This receptor, kinase domain-containing receptor (KDR), is important for endothelial differentiaton and proliferation and is involved in flow-mediated functions of healthy endothelium. It was therefore thought that developing methods for capturing flowing KDR^+ cells onto solid surfaces would be useful as a

potential method to promote endothelialization of vascular grafts via bone marrow-derived progenitors. Cartilage, on the other hand, is not vascularized and the stem cells capable of chondrogenic differentiation remain in the bone marrow, isolated from the cartilage unless the subchondral bone is breached. Thus, for these MSCs an *in vitro* method for enhancing chondrogenic differentiation by providing a physiologically relevant oxygen environment and reducing mass transport gradients was devised and investigated. It was thought that these modifications could also accelerate the *in vitro* generation of stem cell-based neocartilage in a scaffold-free method compatible with ACI. The overall goal of the described studies was to provide insight into methods that could some day be integrated with current or future generations of vascular and chondral interventions.

CHAPTER II

Background

2.1 Tissue Engineering and Bone Marrow-Derived Cells

The area of biomedical research now referred to as tissue engineering arose from aspirations to create replacement tissues for which synthetic implants were deficient and for which organs were not widely available for transplantation [1]. A 2003 National Science Foundation (NSF) report on the emergence of tissue engineering attributes the coining of the term and the conception of the modern notion of the discipline to Yuan-Cheng Fung and his 1985 NSF proposal entitled “Center for the Engineering of Living Tissues,” which went unfunded that year [2]. However, early work done by the team of Robert Langer and Joseph Vacanti – where various cell types were seeded into bioabsorbable scaffolds and implanted into rats [3] – is largely credited as the most influential published study in establishing the field [2]. In their seminal review of the early work that fell under the umbrella of this newly anointed field, Langer and Vacanti defined tissue engineering as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” [1]. They described the three general techniques as 1) using isolated cells or 2) cells seeded within natural or synthetic scaffolds to construct tissues *in vitro*, and 3) using other substances to guide tissue regeneration *in vivo*. The techniques of choice depend on the researcher’s assessments of the tissue’s critical roles in homeostasis, its associated physical and biochemical properties, and the characteristics of the disease or injury responsible for the dysfunctional tissue.

Not surprisingly, early tissue engineering efforts utilized the specialized cells relevant to the tissue of interest – hepatocytes for liver regeneration, dermal fibroblasts

and keratinocytes for skin grafts, endothelium for vascular prostheses, chondrocytes to fill cartilage defects, etc [1]. Isolated cells re-implanted with or without scaffolds can be derived from a separate donor (allogeneic) or collected from the patient's own tissues (autologous). As autologous sources may be limited by the amount that can be safely harvested or may be undesirable due to systemic diseases, allogeneic cells from donors with healthy relevant tissue could provide a means to circumvent these issues of quantity and quality. Unfortunately, implants fashioned from allogeneic cells, like whole organ transplants, are susceptible to immune rejection. Stem and progenitor cells are attractive alternatives as autologous cellular inputs for tissue engineering due to their multilineage potential and the ease of their isolation compared to cells from mature specialized tissues. At the time of Langer and Vacanti's extensive review (1993), the use of stem and progenitor cells for tissue engineering was limited; notable efforts underway were those using bone marrow cells for bone regeneration, hematopoietic stem cells (HSCs) for replacement of blood cells, and skeletal muscle satellite cells for myocardial repair. Since then, the number of tissue engineering endeavors has expanded mightily and increased understanding of stem and progenitor cell biology has provided new opportunities and advanced long-standing efforts [4]. Bone marrow is a reservoir of adult stem and progenitor cells and cells derived from this compartment have been the sources for much of the stem cell-based tissue engineering efforts to date.

Among the cells of the myeloid tissue and stroma of bone marrow are HSCs from which all the cellular components of blood are derived, and multipotent MSCs which are classically characterized by their ability to differentiate into the specialized cells of bone, fat, and cartilage (Fig. 2.1). Less primitive ill-defined vascular progenitors such as EPCs and smooth muscle progenitor cells, which may be derived from HSCs, MSCs, or other precursors, are also present in bone marrow and can be mobilized to peripheral blood through which they home to sites of injury [5, 6]. While biomedical engineers value stem and progenitor cells for their regenerative potential, they can also contribute to pathological conditions. For example, the various subpopulations of bone marrow-derived vascular progenitors may participate in repair of endothelium and neovascularization of ischemic injuries or, alternatively, may contribute to neointimal hyperplasia in atherosclerosis [7, 8]. Elucidating unique identities of vascular progenitors and uncovering the signals that guide the homing of distinct subpopulations to sites of injury will therefore provide invaluable insight to tissue engineers seeking to harness

healing progenitors for vascular interventions. Another common strategy for stem-cell based tissue engineering is to utilize exogenous signals and materials *in vitro* to improve upon naturally observed regenerative responses. MSCs, for instance, participate in repair of osteochondral injuries but the repair tissue lacks the ideal structural and mechanical properties of the original cartilage [9]. Nevertheless, it is effective enough that surgical procedures have been designed to mimic this response by inducing marrow invasion for treatment of pure chondral injuries [10]. However, *in vitro* studies of MSC chondrogenic differentiation give hope that these cells may one day be used to create high-quality tissue engineered replacement cartilage. These physiological responses to and contributions to injuries highlight both the promise and challenge of adapting stem and progenitor cells for use in engineering tissues for which endogenous repair is deficient. Reaching the full potential of any stem cell-based engineered tissue will depend not only on selection of appropriate populations but on the ability to tightly control the response of the cells.

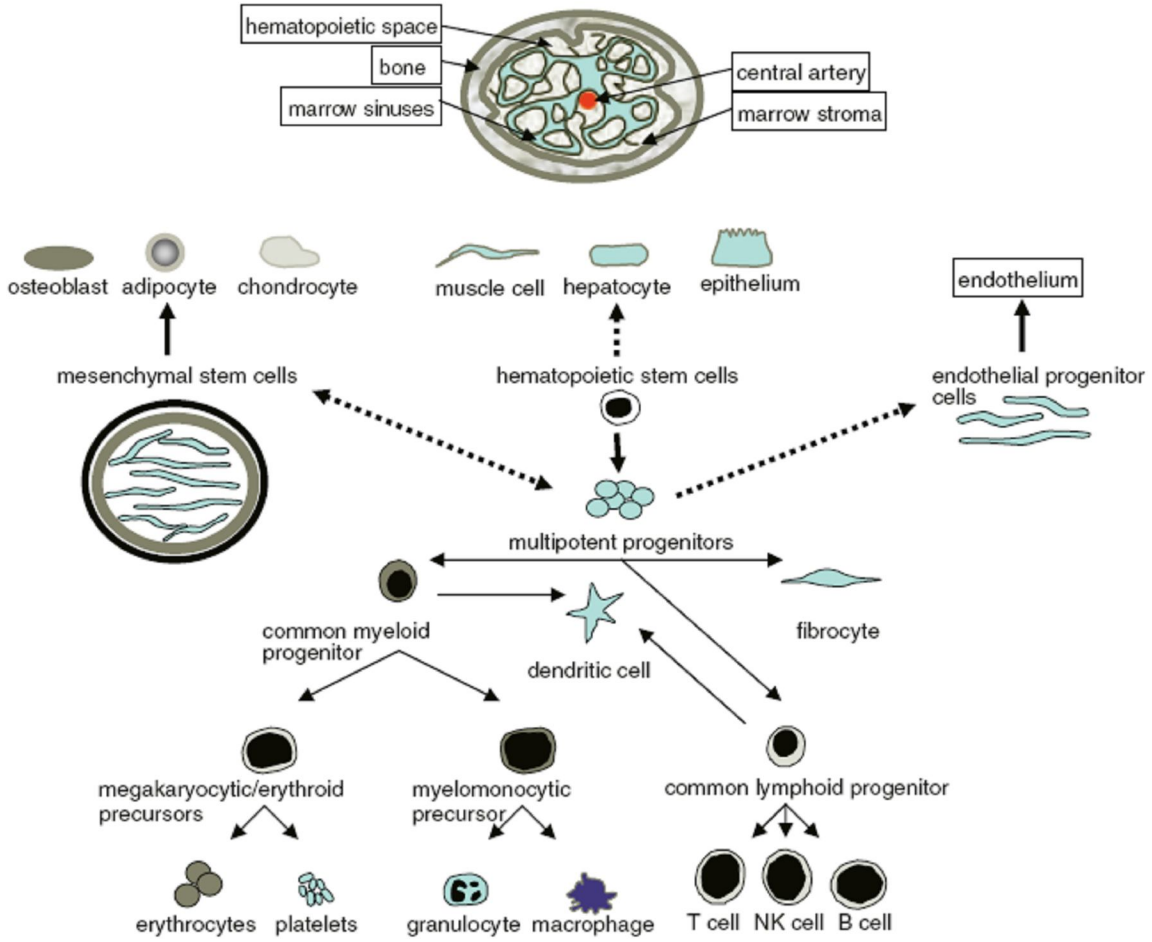

Figure 2.1. Diagram of cross-section of bone and bone marrow cell populations with their associated lineages. Dashed lines indicate controversial areas. Image taken from Wu et al 2007 [11].

2.2 Vascular Engineering

2.2.1 Cardiovascular Disease

Cardiovascular disease has been the annual leading cause of death in the United States for more than a century now (excepting 1918) and is on a trajectory to become the worldwide leader by 2020 [12, 13]. Atherosclerosis, characterized by a thickening and stiffening of vascular walls, is a primary cause of cardiovascular diseases, making vascular bypass surgeries common treatment for the worst cases of coronary artery

disease and peripheral vascular disease. While stents, first of the bare metal variety and more recently drug-eluting, are often used for diseased coronary arteries they are indicated only for vessels that are less than 70% occluded [14]. Additionally, bare metal stents tend to suffer from a relatively high occurrence of restenosis and though drug-eluting stents were introduced to limit the hyperplasia responsible for this problem, many questions still linger, particularly regarding long-term effects [15]. There has been much long-term success utilizing synthetic vascular grafts in the treatment of diseased large-diameter arteries (> 5 mm) but patency has been poor in small-diameter (< 5 mm) vessels such as coronary arteries [16]. The most common grafts used in small-caliber vessel bypass operations are autologous veins or arteries, however, oftentimes patients do not have a suitable source available [17]. While internal mammary arterial grafts are the current “gold standard” for coronary bypass (~90% patency at 10 years), in such operations with vein grafts a patency of only 50% has been observed over 10 years mostly due to changes in the graft resembling atherosclerosis [18, 19]. In cases where autologous vessels are unavailable due to vascular disease, amputation, or previous harvest, synthetic grafts in peripheral vascular bypass operations have demonstrated even lower patency than autologous vein grafts [19]. Synthetic grafts thus far fare worse in terms of patency primarily due to compliance mismatch and thrombogenicity of the material. These inherent properties of the material can lead to early occlusion due to thrombosis and late failure due to intimal hyperplasia [20]. Additionally, current designs often result in incomplete endothelialization upon implantation which certainly does not attenuate the anastomotic intimal hyperplasia prompted by the compliance mismatch [19]. With more than 500,000 small-caliber vessel replacements annually in the United States alone and ~100,000 patients with no usable autologous vessels [13, 17], creating a suitable synthetic alternative continues to be an area of intense research.

2.2.2 Thrombogenicity and Intimal Hyperplasia

Thrombosis is the pathological formation of a thrombus, the result of platelet aggregation mediated by thrombin generation of fibrin. Clots obstructing arterial circulation are generally prompted by rupture of atherosclerotic plaques but thrombosis can also develop in venous circulation for a variety of reasons (Fig. 2.2). The factors leading to vascular thrombosis are described by Virchow’s triad as damage to the endothelial lining of the vessel wall, changes in the pattern of normal blood flow (stasis),

and hypercoagulability [21]. Synthetic grafts are inherently thrombogenic due to the material characteristics of the luminal surface and under low flow conditions the inability of the material to prevent blood coagulation and platelet deposition exacerbates the problem [20]. In uninjured blood vessels, ECs provide an antithrombogenic surface due in part to a glycocalyx layer which prevents platelets adhesion and ectonucleotidases at the luminal surface of ECs which mediate adenosine diphosphate metabolism to adenosine, an inhibitor of platelet aggregation [22]. ECs also prevent platelet adhesion and aggregation through production of nitric oxide (NO) and prostacyclin (PGI_2) by nitric oxide synthase (NOS) and cyclooxygenase (COX) enzymes respectively [23-25]. PGI_2 and NO, both of which are also potent vasodilators, act synergistically to inhibit aggregation, however, only NO has been shown to prevent initial platelet adhesion to surfaces. While platelet aggregation due to impaired endothelial function or vessel wall damage exposing subendothelial matrix proteins can lead to thrombosis, the coagulation cascade may also be initiated by exposure of blood to tissue factor. Quiescent ECs provide protection against this pathway through lack of detectable levels of surface-bound tissue factor and through their surface expression of thrombomodulin and their expression and secretion of tissue factor pathway inhibitor [22].

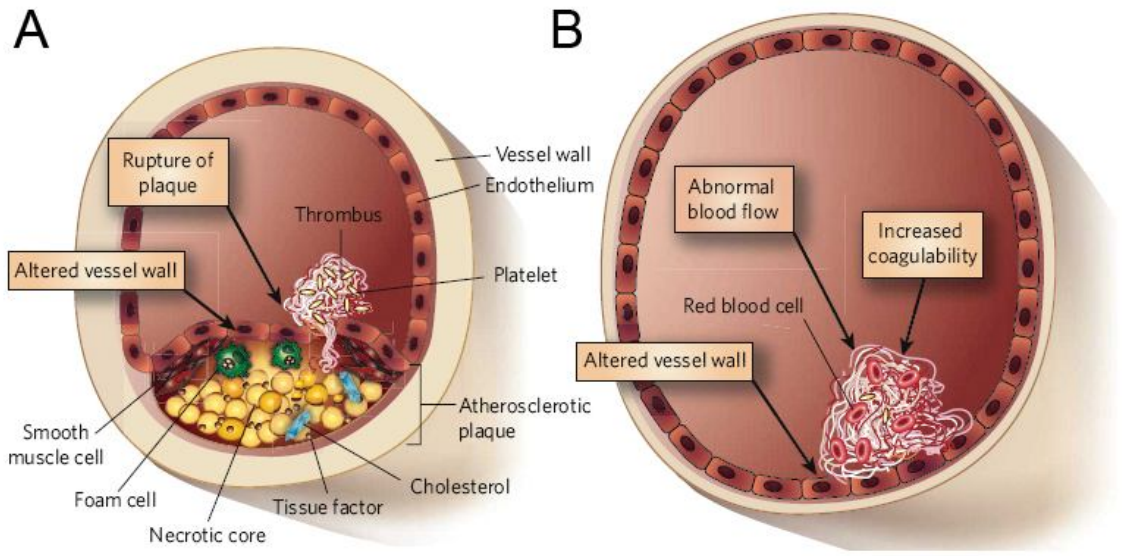

Figure 2.2. Illustration of cross-sections of arteries and veins in pathological conditions. Atherosclerotic plaque rupture generally triggers arterial thrombosis (**A**) while venous thrombosis may occur due to a variety of factors (**B**). Image taken from Mackman, 2008 [21].

Intimal hyperplasia is the thickening of the intima that results in luminal narrowing due primarily from increased smooth muscle cell (SMC) proliferation and migration, extracellular matrix protein deposition, and accumulation of inflammatory cells. It is characteristic of atherosclerotic plaques and often develops following vascular interventions that ultimately fail due to restenosis. In uninjured blood vessels, ECs help maintain the vascular integrity by regulating SMC proliferation and migration. Interestingly, secreted antithrombotic agents such as heparan sulfate, NO, and PGI_2, also inhibit SMC proliferation and migration [26, 27]. Just as vasodilators produced by ECs (NO, PGI_2) inhibit SMC growth, vasoconstrictors (angiotensin II, endothelin, platelet-derived growth factor) typically stimulate SMC growth [27-30]. However, the initial stimulus for SMC proliferation in an arterial injury is due primarily to basic fibroblast growth factor (bFGF) released from damaged ECs and SMCs while platelet-derived growth factor (PDGF) is the major migratory stimulus [26].

In addition to the roles in inhibiting platelet aggregation and SMC proliferation and migration, ECs also have atheroprotective effects through inflammatory response regulation. Neutrophil infiltration has been implicated in atherosclerosis as well as neointimal thickening which is associated with vascular grafts and stents [31-34]. Quiescent ECs are important in preventing leukocyte adhesion and migration, in part through NO and PGI_2 production. NO blocks neutrophil adhesion to ECs possibly by downregulating neutrophil expression of adhesion molecules CD11b and CD18 [35]. Evidence for the role of PGI_2 in inhibition of neutrophil-endothelial interactions points to adhesion molecule-independent mechanisms, perhaps through chemoattractant regulation [36]. As evidenced by its roles in protecting against various atherogenic and thrombogenic stimuli, the EC layer is a vital component of the vessel wall providing a functional barrier to maintain vascular integrity (Fig. 2.3).

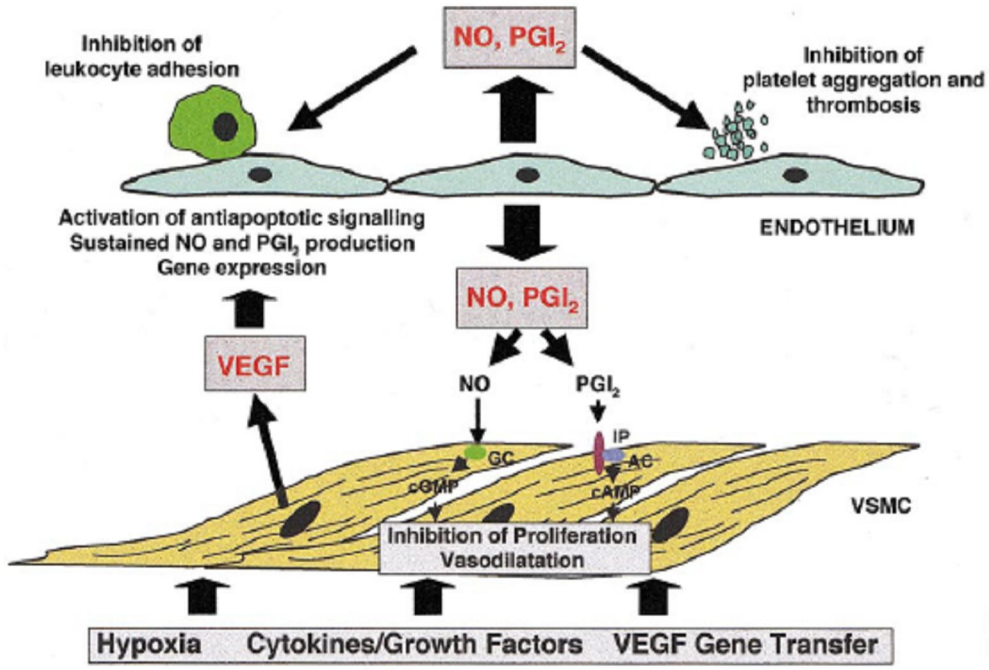

Figure 2.3. Illustration of the multiple vascular protective actions of ECs mediated by their synthesis of NO and PGI_2. Image taken from Zachary, 2001 [37].

2.2.3 Vascular Graft Endothelialization

Two common approaches to improve patency of current prosthetic grafts are coating with antithrombotic agents and directly seeding with ECs. While there has been some short-term success improving graft patency in animal models by coating with heparin [38, 39], the benefits may be limited in the long-term. In addition to providing an antithrombotic surface, heparin inhibits SMC migration and proliferation which may account for the reduced neointimal hyperplasia observed in the short-term [40, 41]. However, heparin can be lost with exposure to blood or plasma and thus far long-term improvements in graft patency have been modest [17, 42, 43]. A major problem affecting long-term patency of prosthetic grafts, especially in humans, is the low occurrence of complete endothelialization and heparin-coating alone may not provide lasting protection against neointimal formation nor improve EC migration from the native vessel. Accordingly, heparin-coating is currently being investigated in combination with surface modifications that increase endothelial cell migration onto and adhesion to grafts. Expanded polytetrafluoroethylene (ePTFE) grafts coated with fibrin, heparin, and bFGF have demonstrated increased surface endothelialization without significant intimal

hyperplasia in a short-term canine model [44]. More recently, bFGF bound to heparinized decellularized grafts has been shown to increase EC proliferation *in vitro* but the performance of the graft *in vivo* has yet to be tested [45]. In another approach, a method for co-immobilizing heparin and the fibronectin cell-binding domain peptide arginine-glycine-aspartate (RGD) to a graft surface demonstrated enhanced EC retention under flow *in vitro* [46]. While coating with heparin and other antithrombotic agents has demonstrated improved graft patency, stimulating and maintaining complete endothelialization even in such modified nonthrombogenic grafts is clearly an ideal goal.

Another biomolecule that has been of particular interest to endothelialization-stimulating strategies, due to its major role in vasculogenesis and angiogenesis, is vascular endothelial growth factor (VEGF). VEGF, through interactions with VEGF receptor-2 (VEGFR-2), is a potent stimulator of EC proliferation and migration [47, 48] as well as an inhibitor of EC apoptosis [49]. Furthermore, it is well documented that VEGF stimulates NO and PGI_2 synthesis by ECs, and that these effects are also mediated at least in part by VEGFR-2, otherwise known as KDR or in mice as fetal liver kinase-1 (Flk-1) [50-54]. For these reasons, some groups have explored the possibility of coating synthetic grafts with matrix-bound VEGF. In a study using ePTFE grafts coated with Matrigel containing VEGF, rats that received these grafts had increased endothelial coverage at one month compared to uncoated and Matrigel-coated grafts but also exhibited greater neointimal hyperplasia [55]. As soluble VEGF was simply physically mixed with the Matrigel (61% laminin, 30% collagen IV), it would presumably be bound via the minor component heparan sulfate proteoglycan-2, meaning its release from the matrix could be mediated by plasmin or other cell-secreted proteases [56]. Interestingly, the results from this study were similar to those observed in pigs receiving ePTFE grafts coated with fibrin to which VEGF was specifically linked in a way that its release would be mediated by cell-secreted proteases [57]. After one month in pigs, these grafts also prompted increased neointimal hyperplasia compared to uncoated grafts but unlike the study in rats, significant improvement in endothelialization was not found. The disappointing results of these studies may be due to other roles of VEGF, such as its proinflammatory action early in vascular injuries. The presence of inflammatory cells was not assessed in the rat study and in the pigs at one month there was no difference in neointimal macrophage counts between VEGF-coated, fibrin-coated, or uncoated grafts. However, VEGF itself is chemotactic for monocytes [58] and others have noted that

increases in VEGF expression after vascular injury promote late neointimal hyperplasia associated with early increases in monocyte recruitment [59]. In addition to the direct action of VEGF, VEGF-induced expression of monocyte chemoattractant protein-1 and other inflammatory cytokines has been implicated in this pathological development [59, 60]. Furthermore, VEGF has been demonstrated to promote chemotaxis of vascular SMCs *in vitro* [61]. It is therefore possible that early monocyte and/or sustained SMC chemoattractant activities of VEGF led to the poor long-term results observed in VEGF-coated grafts in rats and pigs.

Due to humans' reportedly poor ability to self-endothelialize prosthetic grafts [62], inducing EC migration onto grafts *in vivo* is a popular approach. However, these studies utilizing VEGF, a well-known promoter of endothelial function, migration, and survival, highlight the difficulty of preferentially stimulating endothelialization of synthetic vascular grafts *in vivo*. As an alternative, others have focused on improving patency of current grafts by optimizing methods for directly seeding the grafts with autologous ECs or EC-like cells prior to implantation. While there have been a number of methods developed, there also has been no shortage of obstacles, the most common problems due to the source and numbers of ECs obtained and the adhesion and retention of the cells to the graft lumen [20]. Addressing each of these challenges with a singular method remains an elusive goal.

2.2.4 Endothelial Cell Sources

Due to the high immunogenicity of nonautologous ECs, studied sources for graft seeding have been autologous cells including venous ECs, mesothelial cells (MCs), adipose tissue microvascular endothelial cells (MVECs), and peripheral blood-derived EPCs [20, 22]. The first to be used in vascular grafts were venous ECs that were harvested, seeded, and implanted during a single procedure [63]. This method suffered from low cell density initially, followed by poor cell retention upon implantation, but there have been promising results when venous ECs are cultured *in vitro* on the graft prior to implantation [20, 22, 64, 65]. Harvesting venous ECs, however, requires an additional surgery and to obtain a sufficient number of cells necessitates a period of *in vitro* culturing that may take up to five weeks [22, 45]. Such a procedure then is not possible in emergency situations and carries risk of contamination and high costs associated with good manufacturing practice cell culture [22].

MCs isolated from omental adipose tissue have also been studied as a source for graft lining due to high availability, high growth potential, and their similarity to ECs in morphological and functional characteristics [22, 66, 67]. In a canine *in vivo* model, however, MC seeding of ePTFE was found to decrease patency and increase intimal hyperplasia [68]. Furthermore, grafts seeded with human MCs proved to be highly procoagulant in human *ex vivo* and *in vitro* perfusion studies of thrombin and fibrin formation [66]. In the same study, however, MVECs were observed to have comparable antithrombogenic properties to human umbilical vein endothelial cells (HUVECs) and human adult aortic endothelial cells. MVECs are also easily obtainable from subcutaneous fat in high enough numbers to be directly seeded onto grafts and have reportedly shown good resistance to physiological shear stress [66]. Because MVECs can be collected in such high numbers that culturing is unnecessary they have been used for sodding, a pressure seeding method that produces a MVEC-covered graft in a few minutes. MVEC-sodded ePTFE grafts have shown improved patency in canine models but also intimal hyperplasia, which is more progressive in humans [69-71]. The multiple layers of intimal cells was shown to be at least in part due to fibroblast-like contaminants in MVEC isolations and upon further purification, MVEC-sodded grafts showed decreased thrombogenicity and a single layer of cells over the graft at three weeks *in vitro* [69, 72]. Despite improved purification, seeding MVEC on damaged arteries in rabbits after percutaneous transluminal angioplasty resulted in increased intimal hyperplasia and decreased patency [73].

The latest source of cells to be explored for vascular graft lining is EPCs, which originate in the bone marrow and have the capacity to differentiate into ECs. As EPCs circulate in peripheral blood of adults, they are an autologous source that is relatively easy to isolate and do not require an additional operation. Furthermore, a variety of agents has been found to increase EPC mobilization from bone marrow into the peripheral vascular system including granulocyte macrophage-colony stimulating factor, VEGF, erythropoietin, and HMG-CoA reductase inhibitors (statins) [74-77]. However, some ambiguity exists surrounding the identification of EPCs and the capacity of specific populations to differentiate into functional mature ECs (Fig. 2.4). A number of isolation techniques, marker selection criteria, and culture conditions have been employed to generate cells exhibiting mature EC markers, functionality, and morphology from cord blood ($CD34^+$ selected) and peripheral blood mononuclear cells (PBMNCs).

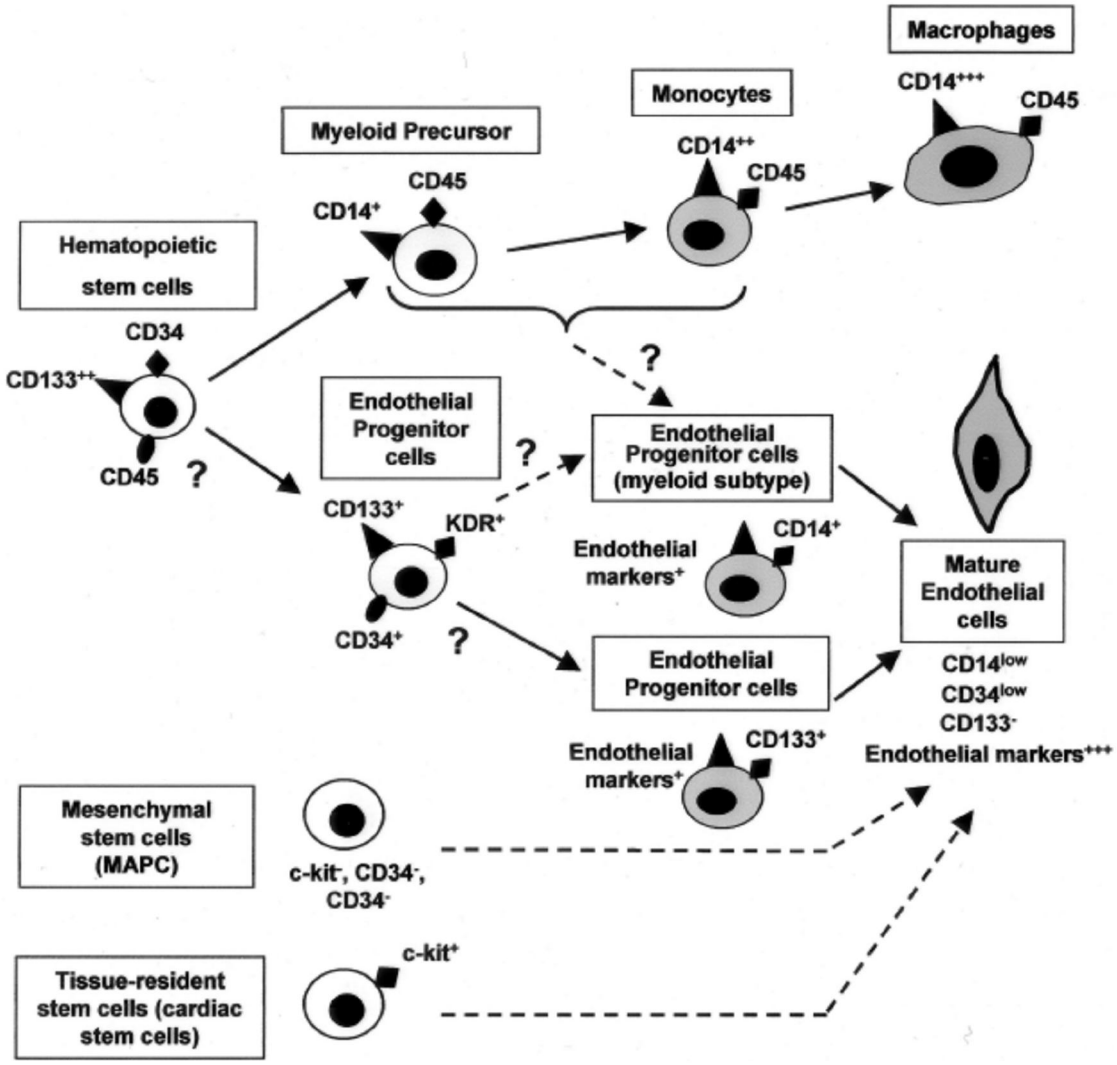

Figure 2.4. Potential origins and differentiation pathways of various stem and progenitor cell populations that can acquire mature EC phenotype. Image taken from Urbich and Dimmeler, 2004 [6].

Asahara et al first described the isolation of EPCs from human PBMNCs using magnetic bead selection of $CD34^+$ cells and the subsequent *in vitro* differentiation of these populations into ECs based on acetylated low density lipoprotein (acLDL) uptake, endothelial specific markers, and functionality [78]. Additionally, the *in vivo* transplantation of human $CD34^+$ PBMNCs or mouse $Flk\text{-}1^+$ PBMNCs in mice and autologous $CD34^+$ PBMNCs in rabbits showed incorporation of the transplanted cells

into the injured tissue at sites of neovascularization. Since then, the early hematopoietic stem cell marker CD133 (or AC133) has been identified as an additional marker for potential EPCs, with $CD34^+/CD133^+$ bone marrow cells demonstrating the ability to differentiate into ECs [79, 80]. Small populations of $CD34^+/KDR^+/CD133^+$ cells have been found in peripheral blood, cord blood, and human fetal liver and are thought to have the capacity to differentiate into ECs [81]. Some groups, including our own lab, have successfully cultured unselected PBMNCs under endothelial growth medium and from adherent populations obtained cells of typical endothelial morphology (termed *late outgrowth EPCs*) that uptake acLDL and express EC markers such as von Willebrand factor, Flk-1/VEGFR-2/KDR, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and vascular endothelial cadherin (VE-cadherin) [82-84]. This method, however, reveals nothing of the lineage of the presumed EPCs and can be highly variable, oftentimes resulting in heterogeneous populations of cells. Because EPCs resembling mature ECs have been generated by this method of adherence-selection and $CD34^+$ and $CD133^+$ circulating cells exist in such small numbers (0.02-0.1% of PBMNCs), Romagnani et al suggested that EPCs can originate from populations other than $CD34^+$ or $CD133^+$ circulating cells [85]. They described a population (0.6-8.5% of leukocytes) expressing the monocyte marker CD14 but having low surface expression of CD34 as another source of peripheral blood-derived EPCs. Interestingly, when KDR^+ cells were isolated from PBMNCs by immunomagnetic sorting, approximately 80% of KDR^+ cells were $CD14^+/CD34^{low}$ and only about 1% were $CD14^-/CD34^+$. Thus, there is still considerable controversy over what constitutes the best marker for EPCs and if there is a definitive phenotypic profile for circulating cells that aid in reendothelialization *in vivo*, it remains a mystery.

Regardless of the lack of a clearly defined phenotype for EPCs, encouraging results have been obtained with various subpopulations used for *in vivo* transplantation. In balloon denuded rabbit carotid arteries, Griese et al transplanted autologous late outgrowth EPCs obtained by culturing the complete population of PBMNCs in endothelial growth medium for two to three weeks [82]. Transplanting EPCs resulted in rapid reendothelialization and reduced neointimal thickening in the rabbits. They also sodded ePTFE grafts with EPCs to 60-70% coverage and although initial retention was poor upon implantation, 40-60% endothelial coverage was found at four weeks indicating that the transplanted EPCs may play a role in recruiting endogenous EPCs or ECs.

Interestingly, human EPCs obtained by adhesion-selection have been shown to express high levels of pro-angiogenic growth factors particularly VEGF and stromal cell-derived factor-1 (SDF-1) and to stimulate the migration of HUVECs [86]. Additionally, exogenous SDF-1 was previously shown to enhance the recruitment and accumulation of culture-expanded human EPCs to ischemic tissue in mice [87]. Another study showed that culture-expanded human EPCs transplanted in mice with ferric chloride-induced arterial thrombosis were recruited to the injured site and that both the cells and their culture supernatants reduced thrombus formation [88]. Recently, human $CD34^-/CD133^+$ and $CD34^+/CD133^+$ PBMNCs expressing equal surface levels of KDR were compared with respect to homing ability and reendothelialization in human limb ischemia and mice carotid artery injuries [7]. In human limbs 120 minutes post-ischemia, there was a decrease in $CD34^+/CD133^+$ cells and an increase in $CD34^-/CD133^+$ cells compared to pre-ischemia; in mice, transplantation of human $CD34^-/CD133^+$ cells resulted in more transplanted cells incorporated into the endothelium at injury sites and greater inhibition of neointimal formation than with $CD34^+/CD133^+$ cell transplantation. In mice with femoral artery injuries, Nowak et al had previously shown that human $KDR^+/CD14^+$ and $Tie\text{-}2^+/CD14^+$ contributed to reendothelialization without increased neointimal formation while no such effect was seen with KDR^- and $Tie\text{-}2^-$ cells [89]. Thus, despite the lack of a well-defined specific phenotype for EPCs, KDR is clearly considered to be an important if not defining marker in the identification of potential EPCs.

A major early benefit of seeding grafts with ECs is the antithrombotic protection provided by the cells' surface properties and their production of NO and PGI_2. The thromboresistance conferred upon various surfaces seeded with ECs is well documented; however, there is limited comparable data for EPCs. In a single one hour study with a baboon arteriovenous shunt model, an ePTFE graft seeded with baboon late outgrowth EPCs resulted in a significant thrombus compared to uncoated ePTFE and ePTFE seeded with HUVECs [90]. This surprising result may be explained in part by reduced NO and PGI_2 production. In segmented polyurethane grafts coated with human late outgrowth EPCs, Shirota et al noted that the cells aligned in the direction of flow and exhibited similar tissue-type plasminogen activator production as HUVECs but produced nearly half as much PGI_2 and contained nearly a third of HUVECs' endothelial NOS (eNOS) protein levels [91]. Their results are similar to those reported by our lab, with late outgrowth EPCs showing alignment with flow and similar coagulation potential as mature

ECs but with undetected eNOS protein expression [84]. While EPCs may indeed produce less NO and PGI_2 than mature ECs, the role of the underlying matrix has yet to be assessed and different adhesive proteins may increase the production of these agents to functionally relevant levels. It is noteworthy that the aforementioned baboon EPC grafts were seeded on type I collagen-coated grafts and it has previously been shown that mature saphenous vein ECs grown on fibronectin-coated ePTFE produced significantly greater PGI_2 than those grown on type I collagen-ePTFE and those on fibronectin/type I collagen-ePTFE [92]. Others have measured similar increases in eNOS gene expression and in levels of NO released from flow-exposed fibronectin-adherent human umbilical cord late outgrowth EPCs as from human aortic ECs [93]. Interestingly, their measurements of eNOS protein expression are not at odds with the aforementioned earlier studies; they also report significantly less eNOS protein expression in the flow-exposed EPCs compared to the ECs.

Despite the increasing evidence of low eNOS expression, one recent study has shown the functional relevance of antithrombotic agents produced by outgrowth EPCs [88]. In this study, static cultures of adhesion-selected human EPCs differentiated on fibronectin showed substantial increases in PGI_2 and NO synthesis and cyclooxygenase-II (COX-II) and inducible NOS protein expressions compared to undifferentiated PBMNCs, while eNOS protein was nearly undetectable in both. The differentiated EPCs and their supernatants were able to block thrombin- and collagen-induced platelet activation and aggregation and to reduce adhesion of flowing platelets to collagen. The inhibitive effects on aggregation were negated by COX inhibitors but not NOS inhibitors, implicating PGI_2 as the primary anti-aggregation agent in this study. PGI_2 is not known to block the initial adhesion of platelets to collagen; however, in this study the COX inhibitor indomethacin reversed the anti-adhesive effects of EPCs and EPC supernatant under flow. This may be explained by an oversight in the perfusion experiments. Indomethacin has been shown to increase superoxide anion production by ECs, which can reduce NO bioavailability [94]. Therefore, the authors showed that the anti-aggregation effects of EPCs were reversed by indomethacin in both the presence and absence of a superoxide radical scavenger, superoxide dismutase. In perfusion studies with indomethacin this enzyme apparently was not included and the effects of NOS inhibitors were not tested; thus an important role for EPC-produced NO should not be ruled out here. Clearly despite the increasing hope being placed on EPCs as a source for vascular engineering

therapies, there is still much to learn not only about selection strategies, but also about the modulation of their expression of atheroprotective agents.

2.2.5 Endothelial Cell Adhesion

Aside from the low availability of autologous ECs and the lengthy period of culturing required to obtain adequate numbers, retention of seeded ECs has been another prominent difficulty with current prosthetic grafts. Methods to improve EC retention on grafts upon exposure to flow include extensive studies of chemical and biological modifications to the luminal surface [46, 95-103], flow preconditioning [104-107], and modification of the ECs themselves [108, 109]. To date, two-staged seeding has demonstrated much better results than single-stage sodding, possibly due to the lack of a good source available in high enough numbers for sodding [62]. For this reason, efforts to increase adhesion have focused on retention of ECs seeded at low density (or subconfluent) and retention of ECs cultured to confluence on the graft surface *in vitro*.

Despite the wide range of approaches to improving EC retention under flow, the majority of studies employ adhesive proteins or cell binding peptides as a substrate in addition to the factor proposed to enhance retention. While improved patency has been achieved in clinical trials of endothelialized grafts with various protein coatings alone (i.e. fibronectin, fibrin glue, RGD-enriched fibrin glue) [64, 110, 111], these studies have used autologous ECs lined confluently onto grafts, thus requiring an extensive culturing period. Additionally, despite aiming for confluence prior to implantation, the washing away of protein coatings has been proposed as a possible limiting factor with adsorbed protein coatings [62]. Of note, adsorbed fibronectin loss from PTFE under flow *in vitro* and *in vivo* has been measured at nearly a third of the initial coating within 30 minutes [112]. Serum or other proteins in solution compete with fibronectin for binding sites and promote desorption of previously adsorbed fibronectin which can be prevented by covalently cross-linking fibronectin to the surface [113, 114]. Furthermore, adsorption of protein results in nonspecific interactions that leave the protein in random conformations and significantly denatured, thus reducing the availability of cell binding sites and biological activity [115, 116]. Not surprisingly then, covalently linking RGD peptide to glass, polyurethane, and ePTFE has been shown to increase EC retention compared to adsorbed fibronectin [98, 100, 117]. In another study, when RGD and fibronectin were

both allowed to adsorb to the surface of PTFE, no difference in initial EC adhesion was observed [103]. Thus, strongly linking and specifically orienting adhesive proteins to surfaces is a promising strategy for enhancing retention by preventing desorption under flow and increasing the availability of cell binding sites.

The incorporation of RGD domains or the use of proteins that possess this binding motif is a common tactic in many of these attempts to improve EC retention under flow and for good reason. Expression of ECs' major receptor for fibronectin's RGD-domain (integrin subunits α_5 and β_1) is significantly upregulated in response to shear stress compared to integrins for other adhesive proteins [118]. The importance of integrin signaling and mechanotransduction in shear stress-dependent eNOS regulation has been documented [119-121]. Similarly, shear stress has been shown to augment PGI_2 release through increased COX-II expression and independently there is evidence for integrin signaling in prostaglandin and PGI_2 release [122-125]. Receptors on the EC luminal surface act as sensors of shear stress and transmit signals through the cytoskeleton to focal adhesion complexes where tension between integrins and extracellular matrix (ECM) propagate flow-mediated signaling cascades [121, 126]. Signaling via these mechanisms is dependent on the number of integrin-fibronectin bonds and possibly on the strength of integrin-ligand bond force [96, 127]. In regards to vascular grafts, strengthening the link between ECs, RGD, and the surface therefore has potential functional benefits beyond increased retention. For example, ECs adherent to surfaces of fibronectin supplemented with RGD peptides immobilized through high-affinity streptavidin/biotin linkages had increased flow-induced NO and PGI_2 compared to ECs on fibronectin alone [128]. Recently, another group has reported other potential benefits of the RGD-containing ECM proteins fibrillin-1 and fibulin-5 for use in vascular grafts [129]. Retention on recombinant RGD-containing fragments of these proteins was not different from fibronectin under low shear stress although it did decrease significantly at higher stresses for both. Interestingly though, these fragments drastically inhibited SMC migration towards potent chemotactic stimuli, VEGF and PDGF, and through EC layers adherent to these fragments. These studies demonstrate the importance of considering the complex interactions and functional consequences in devising unique EC-adhesion promoting surfaces.

Another promising, and not necessarily distinct, approach to improve EC retention is growing the cells directly on the graft lumen under shear stress. Ott and

Ballermann first showed that ECs cultured under long-term exposure to increasing shear stress exhibit enhanced retention when later exposed to high shear stress [106]. Polyurethane grafts seeded with ECs and grown under high shear stress *in vitro* resulted in increased cell retention and decreased neointimal thickness when implanted in rats [104]. Additionally, PTFE grafts endothelialized under slowly increasing shear rates also showed enhanced adhesion at higher shear stress than ECs grown on grafts under static conditions [107]. Interestingly, these EC perfusion cultures also demonstrated increased proliferation rates and enhanced fibronectin expression. Mathur et al showed with their dual ligand system for increasing EC adhesion through avidin-biotin that the effects of flow preconditioning were additive with their system [105]. By augmenting the adhesion through substrate interactions and flow preconditioning, it was demonstrated that the necessary culture time to maintain cell retention may be dramatically reduced.

Recently, some researchers have proposed a type of endothelial seeding under flow that would require no culture time – *in vivo* capture of circulating EPCs to vascular graft or stent surfaces [130, 131]. Like methods designed to induce migration of ECs across anastomoses, this technique is based on the idea of attracting atheroprotective cells without altering their properties through tissue culture. In initial applications of this strategy, anti-CD34 antibodies coated on ePTFE grafts were used to capture $CD34^+$ cells from circulating blood in pigs [131]. The ability of the antibody-coated surfaces to capture circulating cells *in vivo* was clearly demonstrated, however, minimal phenotypic characterization and functionality of the capture cells was presented (lectin binding). In arteriovenous grafts these surfaces displayed rapid cellular coverage (within 3 days) but also exhibited significantly increased intimal hyperplasia at one month. Without detailed characterization of the adherent cells the reasons for this response is unclear. Importantly, Rohde et al have shown that acLDL uptake and lectin binding are insufficient markers to distinguish EPCs from monocytes [132] and thus the grafts may have simply been covered by hyperplasia-promoting monocytes. Additionally, despite the appeal of this strategy it may be limited by the current ambiguity surrounding the definitive identification of EPC populations capable of differentiating into functional mature endothelium.

2.3 Cartilage Engineering

2.3.1 Articular Cartilage Damage and Degenerative Joint Disease

The most prevalent operation on the musculoskeletal system in the U.S. is arthroscopy of the knee [133], whereby a minimally invasive procedure is performed to alleviate joint pain resulting from damaged tissue – generally menisci, ligaments, or cartilage. While arthroscopies of the knee are most often performed for torn menisci and ligaments, retrospective reviews of knee arthroscopies have revealed that hyaline cartilage lesions are observed in approximately 60% of arthroscopies [134, 135]. The most common current treatment for traumatic cartilage injuries is debridement, or the removal of injured tissue, which may relieve pain but does not stimulate repair of the injured tissue [136, 137]. The lack of regenerative capacity of hyaline cartilage of articular joints has led to multiple efforts aimed at stimulating regeneration, surgically transplanting replacement tissue, and tissue engineering neocartilage. One technique used to stimulate regeneration of cartilage is microfracture, an arthroscopic procedure, particularly common for athletes, in which the subchondral bone beneath the damaged cartilage is pierced to induce bone marrow-mediated repair [10]. However, animal studies have shown that this type of procedure results in fibrocartilaginous repair tissue with a different structural composition than the original tissue [138-141]. Two other repair techniques, autologous chondrocyte implantation (ACI) and osteochondral grafts, are common strategies in principle and both have had varied results. For these procedures, osteochondral tissues or chondrocytes from non-load-bearing sites are excised and implanted into lesions. Osteochondral grafts may retain hyaline characteristics but tend to suffer from a lack of integration with the existing tissue at the implant site and significant cell death in the grafts may hamper the procedure [142, 143]. For ACI, the ACs must first be expanded *in vitro* and this results in dedifferentiation with undesirable changes in the cells' phenotypes and in the composition of the matrix they produce [144-146]. Both procedures require a preliminary surgery which itself may cause cartilage degeneration at the donor site resulting in problems later in life [147, 148].

Presenting perhaps a greater challenge than isolated focal trauma is the condition of degenerative joint disease, or osteoarthritis (OA). OA is the most common form of arthritis, a leading cause of chronic pain and disability, and a condition that in the U.S. alone is predicted to affect nearly 60 million individuals by 2020 [149]. Degradation

of articular cartilage is a primary cause of OA and leads to the debilitating pain and dysfunction of the condition that in advanced stages may require surgery to relieve. A history of joint trauma increases the risk of OA [150, 151], for which current microfracture, osteochondral graft, and ACI procedures are not indicated [152]. For patients receiving other arthroscopic procedures, only about half report pain relief and in recent years the effectiveness of arthroscopy for treating OA has been called into question [153-155]. Additionally, as these procedures do not hinder the progression of OA, these patients eventually become candidates for total joint replacement [154]. With nearly 10% of men and 18% of women over 60 years of age worldwide affected [156], developing effective treatments for OA and the joint traumas that contribute to the progression of the disease remains the focus of much international research.

2.3.2 Chondral Defects, Hyaline Cartilage, and Fibrocartilage

Hyaline cartilage is a tissue of low cellularity – cells reportedly make up only 2-10% of the tissue's volume – which functions primarily to provide a low friction surface resistant to compressive forces in synovial joints [157-159]. The articular cartilage covering the ends of bones is a permanent hyaline cartilage with zonal variations in cellular density and matrix structure and distribution (Fig 2.5). Chondral defects, damages to the hyaline articular cartilage surface that do not penetrate the underlying bone, often occur as a result of mechanical trauma. The factors leading to the healing response or lack thereof are the degree of damage to the cellular component and the depth of injury. When the chondrocytes are not damaged, they are able to synthesize new matrix but more commonly the damage encompasses cells and matrix [160]. Due to the avascular nature of articular cartilage, the tissue is not able to regenerate itself after pure chondral damage which is generally categorized as partial or full thickness. In partial thickness defects, the damage may reach the middle zone of the cartilage whereas in full thickness defects, the damage reaches the boundary with the subchondral bone [161]. In each case, the injury's isolation from the blood supply accounts for the inability of the tissue to repair itself. Repair does take place, however, when the depth of damage is such that the subchondral bone is penetrated [162]. In these cases, termed osteochondral defects, blood including stem cells from the marrow fills the defect [9] and this natural response is the basis for microfracture surgery to repair full thickness chondral defects [10]. Unfortunately, this repair tissue is often

fibrocartilage or mixed fibrocartilage and hyaline cartilage [138-141] and this difference in matrix components and structure imparts different mechanical properties on the tissue [163, 164].

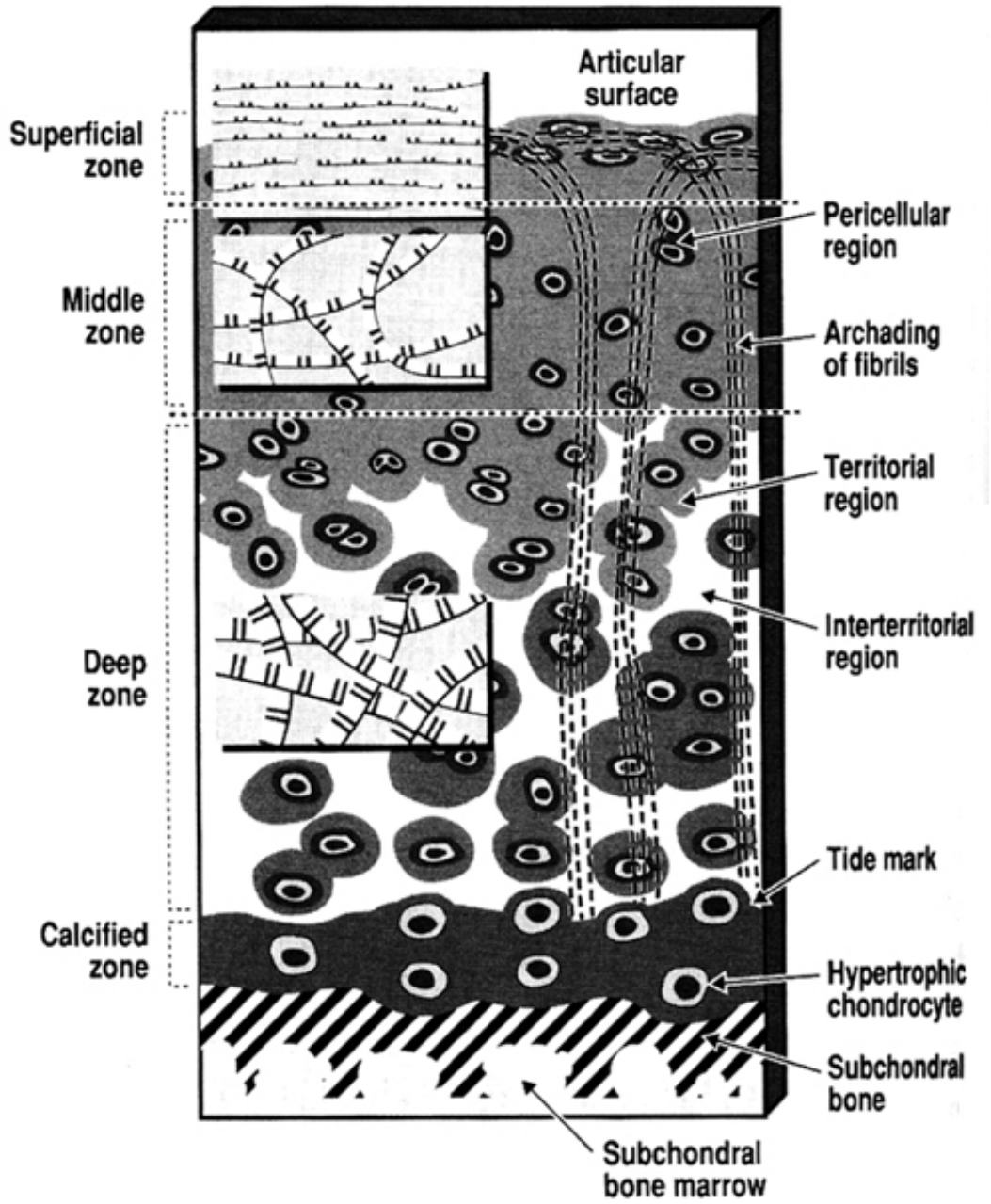

Figure 2.5. Structure and zonal organization of articular cartilage. The variation in cell density across zones is illustrated and the relative diameters and organizations of collagen fibrils are shown as inserts. Partial thickness defects may reach the middle zone, full thickness defects reach the boundary with the subchondral bone, and osteochondral defects penetrate this boundary. Image taken from Poole, 2001 [165].

In the knee, the meniscus is a permanent fibrocartilaginous structure within the synovial joint, between the articular cartilage surfaces of the tibia and the femur, and has provided much of the insight on the composition of fibrocartilage (Fig 2.6). Fibrocartilage is an avascular tissue with a matrix composition resembling fibrous tissue but with some components of hyaline cartilage and, like hyaline cartilage, the substantial matrix is produced by round cells (fibrochondrocytes, in this case) embedded within pericellular lacunae [166]. Water is a major component of both, with fibrocartilage being 55% water and hyaline cartilage being 65-80% water by wet weight but varying by zone [167]. More than 50% of hyaline cartilage dry weight is collagen – with an estimated 86% in the superficial zone down to 67% in the deep zone – compared to over 75% of fibrocartilage dry weight [167, 168]. While the average total collagen content by weight may be similar, a major difference is the predominant type of collagen. In hyaline cartilage an estimated 90-95% of collagen is type II [167] but type I is very lowly expressed in normal ACs [145, 146, 169]. In fibrocartilage, on the other hand, generally 55-65% of the collagen is type I [167] and in the knee meniscus type I may account for a much higher fraction of collagen content [170]. The collagen II that is present as a minor component of fibrocartilage also may undergo different posttranslational modifications compared to that of hyaline cartilage [171]. Furthermore, because collagen II forms smaller diameter fibrils than type I [158], this difference in type predominance is not inconsequential to biomechanical properties of the tissues. Due to differences in collagen types, quantity, and organization, fibrocartilage is estimated to be nearly ten times stiffer than hyaline cartilage [167].

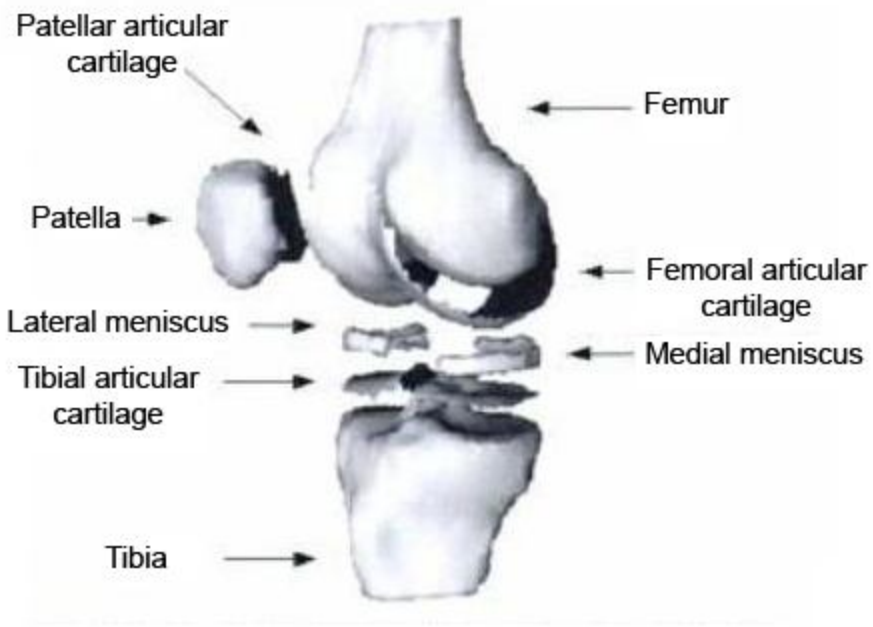

Figure 2.6. Anatomical locations of articular cartilages and fibrocartilages (menisci) of the knee. An exploded view of the components was constructed from the digitization of individual magnetic resonance image slides. Image taken from Abbot et al, 2003 [172] .

While collagen has a large influence on tensile properties, the compressive modulus is directly related to proteoglycan content [158] which may be up to ten times higher in hyaline cartilage than in fibrocartilage [163, 167, 173]. This accounts for the higher water fraction in hyaline cartilage as the hydrophilic nature of sulfated glycosaminoglycan (sGAG) side chains of proteoglycans cause the tissue to swell with water; extended collagen II fibers provide the resistance to the swelling and this dynamic makes for the tissue's unique ability to resist the compressive forces prominent at load-bearing sites [166]. In hyaline cartilage, the large aggregating protein aggrecan, with its chondroitin sulfate and keratan sulfate side chains, reportedly accounts for nearly 90% of the proteoglycan content [174]. Aggrecan is also a component of fibrocartilaginous tissues but it is present in much lower quantities [175] and in meniscus is processed differently with regard to its sGAG side chains and possibly its hyaluronic acid-binding domains than in hyaline cartilage [163, 176]. A comprehensive review of variations in GAG content (including non-sulfated GAGs) measured across studies of meniscus and articular cartilage found that total GAG content was consistently lower in meniscus [168].

The non-sulfated GAG, hyaluronic acid, binds to aggrecan and to collagen fibrils, providing the connection between the two major components of articular cartilage [159]. The proper balance and interaction between collagen II and aggrecan is largely responsible for the mechanical properties of articular cartilage and is something fibrocartilaginous repair tissue can not provide.

2.3.3 Autologous Chondrocyte Implantation

In the 1980s, a technique to repair articular cartilage defects using autologous chondrocytes grown *in vitro* was investigated in rabbit models [177]. In this procedure, a periosteal patch from the tibial surface was placed over the defect and chondrocytes, which had been harvested from a non-load-bearing site and expanded in culture, were injected under this patch. The promising results of these studies led Brittberg et al to perform the procedure on human patients with full-thickness chondral defects and they found mixed results primarily depending on the location of injuries [178]. While follow-ups on this and various other ACI procedures have since demonstrated the procedure as effective treatment for pain relief [142, 179-184], the general process is not without its limitations. Problems include the necessity for an extra operation to retrieve the chondrocytes and the formation of hypertrophic or fibrocartilaginous repair tissue upon implantation [136]. It has been shown that the chondrocyte harvest operation can result in changes in mechanical properties [147] and suggested that the donor site harvest may predispose patients to OA [148]. In a report detailing the two- to nine-year results of the first 101 human subjects to receive ACI, graft hypertrophy associated with the periosteal patch was noted in 40% of the follow-up arthroscopies [180]. Hyaline-like repair tissue was reported in 80% of the 21 biopsies from selected groups that included patients with femoral condyle lesions and osteochondritis dissecans lesions. However, the histological appearance of biopsies from two less successful groups – those patients with patellar lesions and those with multiple lesions which may include patellar lesions – are not reported. While this was a retrospective study without a control group, the results indicated that ACI may be effective for clinical improvement but that the efficacy could vary greatly with type, size, and location of defects within the knee.

In a more recent study of biopsies from 20 ACI patients, six of whom also received osteochondral grafts, at 9- to 34-months post-operation Roberts et al found a much lower incidence of hyaline repair tissue [185]. While nearly all of these patients

presented with femoral condyle lesions, only 22% of the biopsies taken were scored as predominantly hyaline with 30% predominantly fibrocartilage and the rest mixed. Additionally, three of the five specimens that were judged to be predominantly hyaline were taken from patients that had received ACI in conjunction with osteochondral grafts. The authors note, however, that the fibrocartilage specimens were obtained on average eight months earlier than hyaline specimens and so it's possible that hyaline-like repair tissue may develop over time. In another 12-month follow-up of 41 patients who had received ACI for femoral condyle lesions, this same group also found predominantly hyaline tissue in only 10% of the patients while 61% of the biopsies were deemed fibrocartilage [186]. It is still unclear if these low incidences of hyaline repair tissue are due to the time point chosen for follow-up assessment. The average time at which the predominantly hyaline repair tissues were found by Roberts et al was 20 months and they speculated that perhaps the observed fibrocartilaginous repair tissues would eventually transform as well. However, in a study of 40 ACI patients with single femoral condyle lesions, Knutsen et al found predominantly hyaline repair tissue in approximately 20% of biopsies analyzed at 24 months [184]. Another group that evaluated gene expression of repair tissue in four patients at 24-months post-ACI found that the chondrogenic transcription factor (sex determining region Y)-box 9 (SOX9), aggrecan, and collagen II expressions were consistently lower, and substantially so for collagen II and SOX9, than in healthy articular cartilage from multiorgan donors [187]. With such a small study and with patients each suffering from different types of defects, it's difficult to compare directly to the other short-term studies but the results again indicate a consistent lack of hyaline cartilage characteristics even 24 months after ACI. This was also the case in a study where biopsies obtained from six ACI patients within 24 months revealed an abundance of collagen I and limited collagen II in the repair tissue [142]. Moreover, healing of defects in ACI patients from a clinical perspective was slower than in those that received osteochondral grafts.

A recent study that utilized early and late histologic examinations did not lend much credence to the common hypothesis of restoration of classical hyaline tissue with time. In post-ACI analyses consisting of 58 mostly femoral condyle defect patients who had biopsies taken between 8 and 60 months, only 15% of the biopsies were deemed hyaline cartilage with 65% declared fibrocartilage [188]. As part of the analysis, immunohistochemical staining of collagen I, collagen II, and type IIa procollagen was

performed and the patients were grouped into timeframes of biopsies of 8-12 months, 13-24 months, and 30-60 months. The ratio of biopsy area that stained for collagen II compared to type IIa procollagen was significantly increased in later biopsies possibly indicating maturation of repair tissue with time since type IIa procollagen is typically associated with immature developing cartilage. However, on average more than 80% of the area of biopsied repair tissue stained positive for collagen I even in samples grouped from 30 to 60 months post-ACI, while the percentage of area in normal articular cartilage that stained positive for collagen I in this study was only 1.7%. The importance of the repair tissue development can be underscored by the five-year follow-up by Knutsen et al [183]. Of the ACI and microfracture patients whose repair tissue was deemed predominantly hyaline cartilage after 24 months, there were no failures by five years. On the other hand, nearly 20% of those with mixed repair tissue and 20% of those with mostly fibrocartilaginous repair tissue had graft failure within five years of surgery. These results demonstrate the importance of proper matrix development for the successful cellular repair of chondral defects.

The periosteal patch used in ACI requires an additional harvesting operation and is presumed responsible for hypertrophy at implant sites in procedures utilizing this tissue. Thus, studies investigating alternatives such as the use of a porcine collagen I-III patch and matrix-induced autologous chondrocyte implantation (MACI) have been initiated [179, 182, 189]. The porcine collagen I-III patch secured with fibrin glue revealed no difference in repair from a clinical symptom standpoint in initial studies compared to those using a sutured periosteal patch [179]. Out of 19 biopsies taken one year after ACI with the collagen patch, seven were noted as being predominantly hyaline repair tissue. In a separate study, the incidence of hypertrophy was found to be substantially less in patients receiving the collagen patch than in patients receiving a periosteal patch; however, there were cases where repair tissue failed to fuse well with surrounding cartilage in patients receiving the collagen patch, an unobserved phenomenon in their patients receiving the periosteal patch [189]. MACI studies have also utilized a collagen I-III matrix, but one seeded with autologous chondrocytes prior to implantation, and have shown good short-term clinical results [181, 182]. A prospective, randomized study comparing the use of the porcine collagen I-III bilayer as a patch for ACI compared to its use as a component of MACI showed little clinical difference [181]. In each of these studies, however, substantial histological assessment is lacking and the

use of collagen I for MACI raises questions about the nature of the repair tissue since collagen I scaffolds have been shown to induce collagen I expression without sustaining collagen II expression in articular chondrocytes [190, 191]. Perhaps because of some of these unresolved issues, ACI has yet to be established as the gold standard in articular cartilage repair. Consequently, nearly 15 years after the first study in humans, out of more than 400,000 cartilage-related procedures in the U.S. only about 1,500 ACI surgeries were performed [136].

2.3.4 Chondrogenic Cell Sources

The prevalence of fibrocartilaginous repair tissue in ACI is likely due to the dedifferentiation of ACs which occurs during monolayer expansion of these cells. To produce enough cells for ACI procedures, ACs must be grown in culture and current methods for this expansion result in decreased aggrecan expression and collagen I becoming the predominant collagen type over collagen II [145, 146]. This dedifferentiation happens quite rapidly – a recent study comparing the ratio of collagen II to collagen I expression and the ratio of aggrecan to versican expression found decreases of 1,800-fold and 5.6-fold, respectively, over just 10 days in culture [145]. This problem, as well as the desire to avoid unnecessary excision surgeries, has led to the search for alternative cell sources for cartilage repair therapies. A number of cell sources have been shown to contain populations with *in vitro* chondrogenic differentiation ability and thus theoretically may be utilized for autologous cellular therapy to repair cartilage. In humans, these include multilineage MSCs which are found primarily in bone marrow but have also frequently been retrieved from cellular pools in periosteum, adipose tissue, and synovium [192-196]. The term MSC is generally applied to a heterogeneous population of plastic adherent cells that are commonly identified by a panel of surface markers (i.e. $CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD34^-$, $CD45^-$, $CD19^-$, $HLA\text{-}DR^-$) and their ability to differentiate into osteoblasts, adipocytes, and chondroblasts [194, 197]. MSCs selected by various combinations of markers have a well-documented capacity to differentiate into these cell types and there are reports that they can also be driven to other specialized cell types such as tenocytes and myocytes [198, 199].

MSCs from chick embryos and young hatchlings were the first of these stem cells derived from bone marrow to be successfully driven to chondrocytes *in vitro* [200]. The *in vitro* chondrogenic differentiation of postnatal mammalian MSCs isolated from bone

marrow was later described using methods similar to the high-density pellet culture system developed to study mature chondrocytes [201-203]. This system condensed cells into a single aggregate with limited substrate interactions, mimicking limb bud precartilage condensation, and resulted in chondrogenesis when pellets were maintained in serum-free defined medium supplemented with transforming growth factor β1 (TGF-β1). Since these protocols were developed, bone marrow-derived MSCs (BM-MSCs) have been utilized extensively to study neocartilage development and repair cues as well as chondrogenic signaling mechanisms. Due to their involvement in the natural repair of osteochondral defects, BM-MSCs have also been a source of much intrigue to those looking for more robust cartilage regeneration strategies. Furthermore, since OA sufferers do not generally have suitable donor cartilage, BM-MSCs are particularly interesting candidates for autologous cellular repair in these patients. Wakitani et al reported good histological scores when expanded undifferentiated BM-MSCs were implanted within collagen gels in osteoarthritic knees [204]. Clinical outcomes, however, were no different than those that received cell-free gels and specific matrix evaluations were not performed. It seems likely that pre-implantation chondrogenic induction would improve outcomes at least compared to undifferentiated MSCs. An early study comparing the chondrogenic potential of BM-MSCs from OA patients to those of healthy donors found decreased proteoglycan synthesis by chondogenically induced cells from OA patients although collagen II deposition was still detected [205]. BM-MSCs from OA patients were also found to have lower proliferative ability but this can be improved with addition of fibroblast growth factor-2 (FGF-2) to expansion medium [206, 207]. More recent studies have led to renewed optimism regarding the chondrogenic potential of BM-MSCs from OA patients. In polyglycolic acid scaffolds [206] and micromasses supplemented with demineralized bone matrix [208] BM-MSCs from OA patients performed at levels on par with controls. Another study using BM-MSCs from OA patients of various etiologies, found no correlation between age or cause of OA and chondrogenic potential, as assessed by collagen II and aggrecan gene upregulation [209]. Still, while BM-MSCs are widely considered to hold great promise for ACI-type procedures, other sources of MSCs have been increasingly scrutinized with the hope of finding an ideal cellular pool for cartilage regeneration therapies.

The membrane along the outer surface of bones, the periosteum, has long been known to contain undifferentiated mesenchymal cells that can undergo chondrogenesis and as with BM-MSCs, early studies using chick periosteum-derived MSCs (Pe-MSCs) showed the utility of high-density cultures for *in vitro* chondrogenesis of these cells [210]. Due to the reported role of Pe-MSCs in hyaline cartilage repair during fracture healing and the maintenance of chondrogenic ability of these cells with aging [193, 211], Pe-MSCs have drawn interest as a possible source for ACI. Still, substantial comparative studies between human Pe-MSCs and BM-MSCs are lacking although one study indicated less matrix accumulation in differentiated Pe-MSCs [212]. Another recent study notes the limited availability of Pe-MSCs and the burden of an extra invasive surgery and suggests these cells may be more useful for allograft procedures [213]. Additionally, the aforementioned graft hypertrophy attributed to periosteal patches in ACI may be cause for some hesitation to adopt these cells for ACI.

Adipose-derived MSCs (Ad-MSCs) from subcutaneous fat represent an abundant source of multipotent progenitors that are available by comparatively less invasive means [195, 196]. Ad-MSCs, however, have been extensively compared to BM-derived MSCs and have consistently been shown to have a lesser chondrogenic potential by any quantitative comparison [214-222]. It has been suggested that the heterogeneous population of MSCs in the bone marrow may be dominated by osteochondral progenitors while adipogenic progenitors may dominate this pool in adipose tissue [218]. Recent studies have indicated that the reduced chondrogenic potential may be improved by altering current differentiation protocols [223-225] but currently the promise of Ad-MSCs for cartilage regeneration lags behind other sources.

The synovial membrane of joint cavities is a tissue with high regenerative ability that has also been found to house MSCs of chondrogenic potential which can be readily collected during arthroscopy [225, 226]. The condition of synovial chondromatosis prompted the investigation of the *in vitro* chondrogenic potential of synovial membrane cells [227] and later the presence of multilineage MSCs was shown in this tissue in adult humans [192]. Like Pe-MSCs, the chondrogenic potential of synovial membrane-derived MSCs (SM-MSCs) may be unaffected by age [192] and they have a similar proliferative potential as BM-MSCs [226]. In rabbits, SM-MSCs and BM-MSCs differentiated to similar degrees *in vitro* and *in vivo*, and both performed better than Ad-MSCs [228]. Two recent papers by one group have claimed a better chondrogenic potential for human

SM-MSCs compared to BM-MSCs but this was based only on pellet diameter and mass measurements as an indication of matrix accumulation [212, 226]. Djoud et al used BM-MSCs and SM-MSCs from arthritis patients and healthy donors and noted a nonsignificant increase in collagen II and aggrecan expression in SM-MSCs compared to BM-MSCs [229]. Additionally, out of SM-MSC from the eight donors used, three did not show increased collagen II expression upon chondrogenic induction. Conversely, BM-MSCs from all donors, including OA and rheumatoid arthritis patients, demonstrated increased collagen II expression during *in vitro* chondrogenesis. However, a recent study comparing the chondrogenic potential of SM-MSCs from OA patients and healthy donors reported similar ability with regard to cartilage-specific gene expression and protein deposition [230]. Like BM-MSCs, there is ultimately still much unknown about the suitability of SM-MSCs for autologous cellular repair, especially for OA patients.

Recently a number of other purported sources of MSCs with variable chondrogenic ability have been discovered including skeletal muscle, ligaments, articular cartilage, menisci, and even palatine tonsil [212, 231-235]. To successfully harness the healing potential of any of these sources, however, a better understanding of the signals that regulate chondrogenesis is needed. Nude mice models for cell-based cartilage implantation have clearly highlighted this problem. While human ACs have been shown capable of forming stable cartilage when implanted ectopically in nude mice [144, 236], chondrogenically induced human MSCs have not yielded such outcomes. BM-MSCs implanted in the same model suffered from vascular invasion and calcification [236]. SM-MSCs also suffered from vascular invasion, cell death, and generally failed to form stable cartilage [237]. A comparative study using SM-MSCs, Ad-MSCs, and BM-MSCs showed similar deposition of the hypertrophic marker collagen X upon *in vitro* chondrogenic differentiation [225]. SM-MSCs had the lowest alkaline phosphatase (APase) activity, however, and lower levels of APase activity corresponded with less calcification upon implantation in this model; unfortunately, these implants suffered from either a type of dedifferentiation or complete degradation. Ad-MSCs and BM-MSCs in the same system have both been shown to suffer from calcification and vascular invasion [223, 236]. As these researchers concluded, regardless of the source of MSCs the key to utilizing these cells for cartilage repair will be gaining understanding of the control of chondrogenesis and devising methods to stabilize the desired phenotype.

2.3.5 Control of Chondrogenic Differentiation

Perhaps the most difficult challenge facing the use of MSCs for cartilage regeneration lies in achieving a stable articular cartilage phenotype. This is not surprising given how little is known about the fetal development of permanent articular cartilage. In vertebrate skeletogenesis, aggregated mesenchymal cells give rise to a transient cartilage that develops the skeletal structure before being replaced by bone. Studies on embryonic chick and mouse limb bud development have provided much of the insight on these processes of condensation, chondrogenesis, and endochondral ossification. The expression patterns of these events and the transitions between them are controlled by intricately coordinated cell-cell interactions, cell-matrix interactions, external signaling molecules and transcription factors (Fig. 2.7).

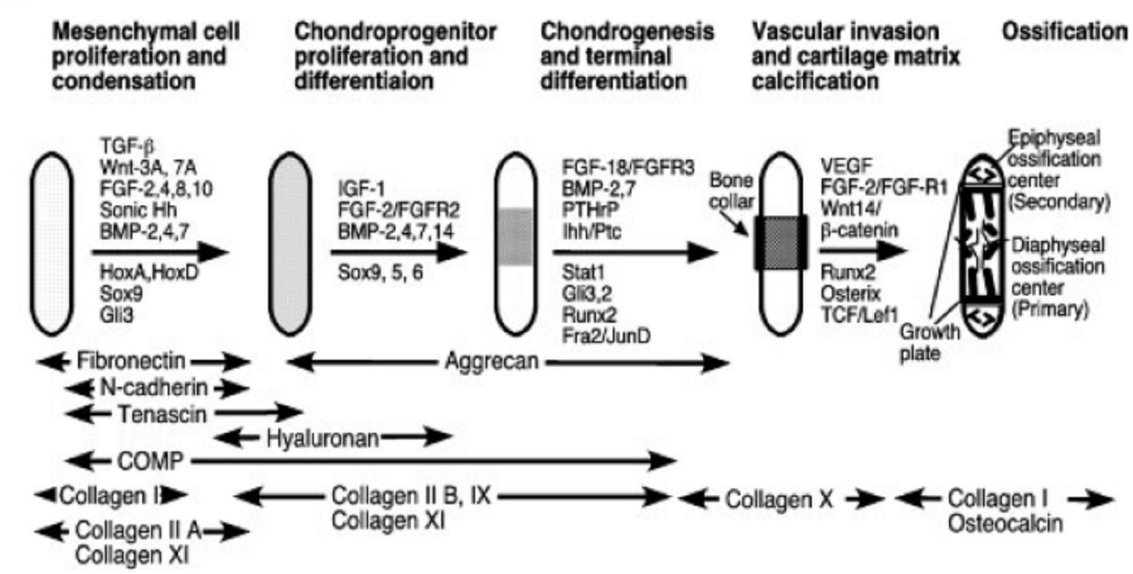

Figure 2.7. The sequence of events of chondrogenesis during the development of long bones. Some of the known growth and differentiation factors involved in the transition between stages are shown above the arrows and the associated transcription factors are noted below the arrows. Some of the known characteristic ECM proteins of the stages are shown below the stages. Image taken from Goldring et al 2006 [238].

During condensation, prechondrogenic mesenchymal cell aggregates form, mediated in part by cell adhesion molecules such as neural cell adhesion molecule (N-CAM) and neural cadherin (N-cadherin) and ECM components such as fibronectin, collagen I, and syndecan [239]. Among the early cell-cell contacts formed, it has been

suggested that the calcium-dependent N-cadherin initiates mesenchymal condensation and calcium-independent N-CAM stabilizes the aggregation and that both are essential for initiation of chondrogenesis of these cells via condensation but are lost during differentiaton [240-242]. The expressions of N-CAM and N-cadherin, as well as fibronectin, have been found to be increased by various members of the TGF-β superfamily (TGF-βs, bone morphogenetic protein-2 (BMP-2), activin) in mouse [243, 244] and chick embryonic mesenchymal cells *in vitro* [245-247]. Fibronectin, as well as collagen I, increases in the limb bud during condensation but fibronectin gene and protein expression decreases and the structure changes during chondrogenesis [248-251] and collagen I synthesis is replaced by collagen II synthesis [248, 252, 253]. Syndecans are also transiently expressed and required for condensation to occur and, through interactions with GAGs, fibronectin and collagen I, may help maintain the shape of the growing limb bud by enhancing interactions between the ECM and the mesenchymal cells prior to the onset of chondrogenesis [254-256]. This intimate arrangement of the mesenchymal cells is necessary but temporary and as condensation shifts to chondrogenesis other ECM components such as tenascin may guide this transition. Tenascin has been speculated to promote the rounding of mesenchymal cells by disrupting the cell integrin-fibronectin interactions that enhance condensation but inhibit chondrogenesis [257-260]. Expression of tenascin, like N-CAM, N-cadherin, and fibronectin, is upregulated by TGF-βs [243, 245]. Intracellularly, the nuclear transcription factor SOX9 has been identified as an essential regulator of limb bud mesenchymal condensation. When SOX9 was inactivated in undifferentiated mesenchymal cells of mice embryos prior to condensation, the limb buds did not develop normal mesenchymal condensations and no cartilage or bone formed while apoptosis in the limb buds increased [261]. The authors of this study also noted that N-cadherin and N-CAM expression levels appeared unaffected by SOX9 inactivation and speculated that SOX9 controls other essential genes for condensation. In addition to SOX9, the transcription factor runt-related transcription factor 2/core-binding factor α1 (Runx2/Cbfa1) begins to be expressed during condensation and it is the balance of their activities – under tight regulation by TGF-βs, BMPs, FGFs, and Wnt signaling molecules – that largely dictates the subsequent stages of limb development [262]. Moreover, SOX9 itself can inhibit Runx2/Cbfa1 and it has been hypothesized that this repressor activity early in

mesenchymal condensations is responsible for the subsequent chondrogenic lineage determination during skeletogenesis [263].

Following condensation, the prechondrogenic mesenchymal cells differentiate to create a cartilaginous template for the formation of long bones and the joints. The cartilaginous skeleton is formed as chondrocytes proliferate, secrete cartilage-specific ECM (aggrecan, cartilage oligomeric matrix protein (COMP), collagen types II, IX, and XI), and organize into columns extending the skeletal template [239]. The transcription factor hypoxia-inducible factor-1α (HIF-1α) has been identified as an important regulator for this transition from precartilage condensations to overt differentiation [264, 265]. The avascular mesenchymal condensations that form create a hypoxic environment that stabilizes HIF-1α and allows it to promote target genes involved in chondrogenesis. Most importantly, HIF-1α may upregulate expression of the nuclear transcription factor SOX9 by binding directly to its promoter [265]. SOX9 is the key transcriptional regulator in this stage and was the first identified essential transcription factor for chondrogenesis [266]. Mutations in the SOX9 gene in humans lead to campomelic dysplasia, a usually lethal skeletal malformation disease [267]. When SOX9 was inactivated during or after the formation of mesenchymal condensations in mice embryos, differentiation and proliferation in the limb buds was substantially reduced and ultimately cartilage failed to form [261]. SOX9 inactivation also revealed that it is required for expression of the essential transcription factors SOX5 and SOX6 [261] and together these three SOX transcription factors promote the expression of genes for the primary cartilage matrix proteins collagen II and aggrecan [268-270], as well as COMP [270, 271], and collagen XI [269, 270, 272]. In terms of regulation of the genes for collagen II, aggrecan, and collagen XI it has recently been shown that SOX5 and SOX6 increase the binding affinity of SOX9 to their enhancers and thus the activation of SOX5 and SOX6 by SOX9 serves as a potent positive self-regulatory mechanism [269]. Additionally, SOX5 and SOX6 may differentially regulate other SOX9 targets. For example, SOX9 activates the collagen IX gene but SOX5 and SOX6 have been reported to either have no effect or to negatively regulate expression of this gene [273, 274]. Although there are indications that SOX5 and SOX6 may be important for the activation of genes for other cartilage matrix proteins [270, 275], the exact role of the SOX trio for many chondrocyte genes is yet to be determined. However, recently it has been shown that the SOX trio does cooperate to directly upregulate parathyroid hormone-related protein (PTHrP) [276], an inhibitor of

chondrocyte hypertrophy and calcification [277]; in this way the SOX trio acts as a negative regulator of the subsequent stages of skeletogenesis.

While some cartilage at the ends of long bone, the epiphyses, remains as permanent articular cartilage postnatally, the rest is transient cartilage that is replaced by bone during the process of endochondral ossification. As the chondrocytes cease proliferation they undergo hypertrophy which further extends the cartilaginous skeleton and provides signals to drive vascularization and calcification [239]. Hypertrophy is characterized by increased cell size and a switch in collagen type expression and synthesis from predominantly fibrillar type II to predominantly short chain type X [278-280]. This shift from proliferating chondrocytes to hypertrophic chondrocytes is accompanied by a major shift in the balance of the expressions of SOX9 and Runx2/Cbfa1 [238]. Various studies utilizing *in situ* hybridization in mice embryos have identified high expression levels of Runx2/Cbfa1 from prehypertrophic through terminal hypertrophic chondrocytes with little detected in resting or proliferating chondrocytes [281-283], whereas the opposite pattern of distribution is observed for SOX9 [284], as well as SOX5 and SOX6 [268]. Runx2/Cbfa1 is the essential transcription factor promoting hypertrophy and directly activating expression of collagen X [281-283, 285-288]. One of the primary enzymes associated with biomineralization, APase, also becomes prominent in the hypertrophic zone and Runx2/Cbfa1 participates in regulation of its expression and activity as well [281, 289, 290]. In transgenic mice studies, when Runx2/Cbfa1 was overexpressed or maintained in normally nonhypertrophic chondrocytes under control of a collagen II gene promoter and enhancer, chondrocyte hypertrophy was increased and observed in atypical locations. These transgenic embryos' skeletons underwent more rapid endochondral ossification with extensive abnormal mineralization [283, 287]. On the other hand, disruption of the Runx2/Cbfa1 gene or specific inactivation in chondrocytes of transgenic mice resulted in embryos with stunted endochondral ossification that maintained a mostly cartilaginous skeleton with high collagen II expression, little collagen X expression, and virtually no APase activity [287, 291].

In addition to its roles regulating collagen X and APase, Runx2/Cbfa1 promotes the expression of other factors in hypertrophic chondrocytes such as matrix metalloprotease-13 (MMP-13) [281, 291] and VEGF [292], the secretion of which aid in altering the surrounding ECM and promoting vascularization. MMP-13 becomes

expressed in terminal hypertrophic chondrocytes and, along with other MMPs such as MMP-9 expressed by invading chondroclasts, is necessary to degrade the cartilaginous matrix just prior to vascularization [293-296]. VEGF expression, which is not detected in resting or proliferating chondrocytes, increases as prehypertrophic chondrocytes hypertrophy [292, 295, 297] and the secreted growth factor is thought to bind to ECM before its release is mediated by MMP-9 [294]. VEGF is essential for vascular invasion of the growth plate and inactivation of VEGF or MMP-9 leads to an increased hypertrophic zone due to impaired hypertrophic chondrocyte apoptosis, cartilage matrix resorption, and mineralization associated with vascular invasion [295, 298]. The infiltration of blood vessels, chondroclasts, and osteoblasts thus coordinates with death of the hypertrophic chondrocytes and the conversion of the cartilaginous template to skeletal bone.

Already much has been revealed about the signals regulating chondrocyte differentiation, maturation, hypertrophy, and endochondral ossification, but very little is known about how permanent ACs develop and maintain a stable phenotype resisting maturation and hypertrophy. Eames et al have proposed one model for the regulation of fate decisions of osteochondral progenitors based on their study of chick cranial skeletogenesis, in which three distinct areas of mesenchymal condensation form tissues of either transient cartilage or permanent cartilage, or undergo direct conversion to bone [299]. During embryonic development, the areas that would be transient cartilage expressed SOX9 *and* Runx2/Cbfa1 as has been reported in limb bud mesenchymal condensations [261, 285, 300]. In contrast, those areas that would develop into permanent cartilage expressed only SOX9 at detectable levels, while only Runx2/Cbfa1 was visualized in those areas that would undergo direct osteogenesis. Due to these observations and SOX9 and Runx2/Cbfa1 misexpression experiments, they propose that permanent chondrocytes develop from mesenchymal cells where SOX9 expression is constitutively increased while Runx2/Cbfa1 expression is maintained low. However, as noted, both SOX9 and Runx2/Cbfa1 are expressed in limb bud mesenchymal condensations and when SOX9 was inactivated in mesenchymal cells prior to condensation Runx2/Cbfa1 was not detected in limb buds [261]. So in addition to the question of signals are questions regarding the source of the permanent ACs. It may be that portions of the epiphyseal ends do not ossify and that these cells are part of the original cartilaginous template. This has been traditional thinking but some have

proposed that permanent ACs develop from some population of mesenchymal cells that are not a part of this initial anlagen [301]. During the formation of the cartilaginous skeleton, an interzone of flattened cells forms at the future joint site where cavitation occurs to create the synovial space [302]. On either side of this space and perpendicular to the developing columns of transient cartilage will be the articular surface of the epiphysis. It was recently shown that mesenchymal cells surrounding the future joint migrate into the cartilaginous skeleton and contribute to the formation of the interzone and it was suggested that permanent ACs may be derived from these cells [303]. The surrounding perichondrium has been suggested to promote the hypoxic environment that develops at the interzone, resulting in the stabilization of HIF-1α here [264]. If permanent cartilage indeed develops from these newly arrived cells, this hypoxic environment could promote the chondrogenesis at the joint site in a manner similar to its role in the initial limb bud differentiation.

Little is known of unique signals involved in permanent articular cartilage development but the transcription factor ets related gene (ERG) was found in limb bud condensations of chick embryos [304] and it was later demonstrated that developing chicks express a variant, termed C-1-1, which persists in the permanent articular chondrocytes of the epiphyseal ends [305]. This variant was also shown to block hypertrophy and endochondral ossification in developing chick limbs and inhibit APase activity and mineralization in C-1-1 transfected chondrocytes *in vitro*. Notably, C-1-1 is thought to exert its effects through a mechanism independent of Runx2/Cbfa1 modulation [306]. Runx2/Cbfa1 is a powerful negative regulator of the formation of permanent cartilage as overexpression in transgenic mice leads to hypertrophic chondrocytes and bone formation in locations that are normally permanent cartilage [287]. While both transcription factors are present in mesenchymal condensations, Runx2/Cbfa1 overexpression leads to downregulation of C-1-1 but overexpression of C-1-1 is claimed to have not resulted in significant changes to endogenous Runx2/Cbfa1 expression [306].

Most of the studies designed to elucidate mechanisms involved in promoting the permanent AC phenotype have used chick development models. The usefulness of developing chick embryos for studying the formation of permanent articular cartilage in humans has been questioned [307] because of the prevalence of collagen I and the fibrocartilage nature of postnatal and mature chicken articular cartilage [308-311].

Further complicating this issue is that a variant of human ERG homologous to C-1-1 was identified while a variant of mouse ERG homologous to C-1-1 could not be identified [312]. Additionally, in mice ERG was found only in epiphyseal chondrocytes and not in the growth plate as it was in chicks. However, ERG expression in both chicks and mice was promoted by growth/differentiation factor-5 (GDF-5) although the specific variants affected are not known. The authors hypothesized that GDF-5 could act on chondrogenic cells at the interzone through ERG or its variants to promote a permanent articular phenotype but this has not been proven.

The utility of adult human MSCs for cartilage regeneration therapy may ultimately be limited by this lack of understanding regarding the formation of permanent articular cartilage. Directing these cells to transiently obtain expression profiles consistent with non-hypertrophic chondrocytes *in vitro*, however, is just as complex. Regarding *in vitro* chondrogenic ability, BM-MSCs are the most studied and best characterized of human MSCs by far. The methods for inducing *in vitro* chondrogenesis are based on recapitulating known signals for limb bud chondrogenesis but to date these cells have not shown the ability to obtain the unique chondrogenic expression profiles associated with the sequential stages defined in development studies. The first protocols developed utilized the signals that arise from aggregate formation and provided exogenous TGF-β_1 or other members of the TGF-β superfamily to promote chondrogenesis [201-203, 313]. While this results in chondrogenesis as determined by expression of typical cartilage markers such as collagen II and aggrecan, the differentiated MSCs also tend to substantially upregulate markers associated with hypertrophy and endochondral ossification. Additionally, the temporal upregulation of these genes in human BM-MSCs under current *in vitro* conditions is different than that of embryonic mesenchymal cells in skeletogenesis. For example, collagen II expression precedes collagen X expression in the developing limb bud and the switch in collagen type expression and synthesis is a defining characteristic of the hypertrophic stage [278-280]. During *in vitro* chondrogenic differentiation of human BM-MSCs, collagen X gene and protein expressions have consistently been reported to increase in conjunction with or even earlier than collagen II [202, 236, 314-319]. Similarly, collagen I has a well-defined temporal expression pattern in skeletogenesis. It is highly expressed in embryonic mesenchymal cells prior to chondrogenesis before being replaced with collagen II [248, 252, 253]. Collagen I again becomes the dominant collagen type deposited during endochondral ossification [253,

311, 320] and although it is present in adult chicken articular cartilage [308], it is nearly absent in healthy adult human articular cartilage [145, 146, 169]. Collagen I is highly expressed in human BM-MSCs [321] and in pellet cultures of these cells, collagen I gene expression has been noted to increase throughout chondrogenic induction or to slightly decrease early but increase later, simultaneously with collagen II and collagen X [236, 315, 316]. Premature gene expressions of VEGF [319] and MMP-13 [236, 319] have also been observed in human BM-MSCs differentiated *in vitro*.

While these differences in chondrogenic expression patterns may be inherent to BM-MSC differentiation it's also possible that the myriad *in vitro* conditions devised have yet to provide appropriate environmental cues to result in a sequential progression through chondrogenic stages more reminiscent of that seen in development. In evaluating the delivery of these signals, the platform for *in vitro* induction must also be considered. The most common culture platform for chondrogenesis is high cell density pellet cultures which physically forces the aggregation stage needed for the onset of chondrogenic differentiation. This has been an invaluable platform for studying MSC chondrogenesis but the physical dimensions of macroscopic pellets may hinder the kind of ordered, coordinated differentiation which would be essential for tissue engineering uses. The heterogeneity of proteoglycan accumulation and collagen II synthesis in MSC pellet cultures has been well-documented if not consistent in spatial distribution [202, 203, 313, 314, 316, 317, 322-324]. It appears that in addition to the temporal disorder of gene expression in BM-MSC chondrogenesis, current techniques also result in a spatially nonuniform differentiation. This uneven chondrogenic response routinely persists over the course of studies – often for as long as three or four weeks. So while increasing knowledge of the signals that promote permanent articular cartilage should improve our MSC chondrogenesis protocols, modifications to culture platforms should also be useful. Currently it remains that we are unable to achieve finely controlled familiar AC genotypes and phenotypes from MSCs, even temporarily.

CHAPTER III

Anti-KDR Antibody Endothelial Capture Surfaces[1]

3.1 Abstract

In humans, self-endothelialization of synthetic grafts is severely limited but a recent interesting idea is to attract EPCs from peripheral blood onto grafts via antibodies directed at proposed EPC markers. Results with anti-CD34 antibodies have shown some promise but it is unclear if CD34 is the best marker for cells with re-endothelializing potential. Much evidence points to KDR as an important indicator of endothelial potential if not a definitive marker. Because KDR is not an adhesion molecule (as CD34), we first demonstrated the ability to use adsorbed and protein G-oriented antibody to this receptor to capture flowing cells onto a solid surface. Using ECs (KDR^+) and SMCs (KDR^-), we show in a model system under low shear rates the ability to selectively capture cells by this receptor. Capture of cells via anti-KDR monoclonal antibody (mAb) was observed at shear rates of 50 s^{-1} and this was shown to be due to specific interactions with the antigen-binding domains. Compared to adsorbed anti-KDR mAb, protein G-oriented anti-KDR mAb caused increased cell capture despite significantly less total antibody density on the surface. When KDR^+ cells were mixed with KDR^- cells in physiologically relevant ratios, capture rates of KDR^+ cells were unaffected and capture of KDR^- cells was not observed. Our results indicate that selective capture of flowing KDR^+ cells is feasible and that efficiency of capture is unaffected by concomitant flow of cells lacking the receptor but significantly increased by orienting the antibody. The results of capture via KDR shown here indicate this receptor may be a suitable alternative to CD34 as a target molecule in antibody-coated vascular devices.

[1] This research was originally published in *Tissue Engineering Part C: Methods*. Markway, B.D., McCarty, O.J., Marzec, U.M., Courtman, D.W., Hanson, S.R., and Hinds, M.T., "Capture of Flowing Endothelial Cells Using Surface-Immobilized Anti-KDR Antibody," *Tissue Eng Part C Methods*.

3.2 Introduction

The success of synthetic small-diameter (< 5 mm) vascular grafts in the treatment of coronary artery disease and peripheral vascular disease has so far been limited in the short-term by thrombosis and more dramatically in the long-term by intimal hyperplasia [20]. The luminal surfaces of grafts are thrombogenic and under low flow conditions the inability of the material to prevent blood coagulation and platelet deposition exacerbates the problem [20]. Additionally, as humans have reportedly shown poor ability to self-endothelialize prosthetic grafts, the anastomotic intimal hyperplasia prompted by compliance mismatch is not well-regulated [62]. Because the EC lining is a key component to inhibiting thrombosis and intimal hyperplasia in native vessels, endothelialization of synthetic grafts is a popular and promising tissue engineering approach to improve patency. Common problems encountered include the absence of a source of sufficient numbers of ECs for rapid use, the necessity of a second operation for harvesting, and the retention of the cells after implantation into a flow-exposed environment [20, 22]. One method that could provide an off-the-shelf solution that would avoid such problems is to engineer graft surfaces that promote endothelialization upon implantation. Impregnating grafts with factors that stimulate ingrowth from ECs at the anastomoses is such a strategy and one that has long been investigated [44, 55, 57]. A more recently conceived approach, and one for which variations of the original efforts have been increasing, is to attract the circulating EPCs from flowing blood to the surface. The first of these studies, and the most developed method so far, used antibodies directed at CD34 to capture cells onto luminal surfaces of vascular devices [130, 131]. *In vivo* results clearly indicated accelerated cellular coverage of these surfaces but given mixed clinical outcomes, questions remain about the selectivity of the method in general as well as the choice of CD34 as the targeted antigen. The purpose of this research was to evaluate the antibody-mediated capture of flowing cells *in vitro*, and specifically to study the selectivity and efficiency of capture when antibodies are immobilized via methods that impart distinctly different conformations on the antigen-binding domains. Additionally, the VEGF receptor KDR was chosen as a target molecule due to its evident importance in identifying EPCs and its potential to mediate atheroprotective benefits at sites susceptible to thrombosis and neointimal hyperplasia, such as surfaces of vascular grafts.

3.3 Background

3.3.1 Antibody-Mediated EPC Capture Devices

The latest source of cells to be explored for vascular graft lining is EPCs, which originate in the bone marrow and have the capacity to differentiate into ECs. As EPCs circulate in the peripheral blood of adults, they are an autologous source that is relatively easy to collect and do not require an additional operation. Unfortunately, obtaining ample numbers of appropriately differentiated cells demands similarly long culturing periods as explanted ECs. To circumvent this problem, recent studies have focused on attracting EPCs from the circulation onto implanted blood-contacting surfaces by incorporating antibodies directed at proposed EPC markers. Anti-CD34 antibody has been used to capture $CD34^+$ cells from circulating blood *in vivo* but little was reported about the dynamics and efficiency of capture and the phenotypic fate of captured cells [130, 131]. In porcine arteriovenous ePTFE grafts anti-CD34 antibody-coated surfaces resulted in rapid cellular coverage but significantly increased neointimal hyperplasia. While the surfaces of the grafts clearly attracted cells to a degree not seen in uncoated grafts, the methods presented to identify the cells were not stringent enough to distinguish EPCs from monocytes [132]. Additionally, specificity of capture is not clear from the presented data. Still this same technology was applied to stents and implanted in 16 patients in a clinical trial that deemed them safe as of nine months but the levels of luminal narrowing was similar to those reported for bare metal stents [130]. With little characterization of the captured cells in these studies, the reasons for the lack of protective effects against hyperplasia are unclear. As noted by the authors, there are multiple possibilities including a lack of physiological matrix for the captured cells, undesired paracrine effects, and the multipotential nature of $CD34^+$ cells. Of particular concern regarding the targeting of $CD34^+$ cells for vascular grafts is their potential to differentiate into smooth muscle cells [325].

3.3.2 KDR

Much ambiguity still exists surrounding the identification of EPCs and the capacity of specific populations to differentiate into functional mature ECs. They are generally regarded to express some combination of markers for both HSCs (i.e. CD34) and ECs and recent evidence points to KDR as one of the more important indicators of

endothelial potential [7, 78, 81, 85, 89]. This is not surprising given its critical role in vasculogenesis and in mediating many of the functions of ECs through interactions with VEGF. Mice embryos deficient in Flk-1, the mouse homologue of KDR, die *in utero* due to their inability to form blood vessels and mature endothelium [326, 327] whereas CD34-deficient mice embryos exhibit delayed hematopoiesis but survive postnatally with apparently normal vasculature [328]. Furthermore, while the functions of CD34, particularly with regard to ECs, remain unclear [329], KDR is known to modulate many functions of ECs including stimulating proliferation and migration [47, 48] and inhibiting apoptosis [49]. Additionally, the VEGF-induced upregulation and activity of enzymes eNOS and COX-II are promoted through KDR [50-54]. In ECs, eNOS and COX-II synthesize the potent vasodilators NO and PGI_2, respectively, which play critical roles in preventing platelet adhesion and aggregation and SMC proliferation and migration [23-25, 27].

In addition to its role as a growth factor receptor, the ligand-independent activation of KDR was recently found to be involved with a mechanosensory complex that mediates responses to shear stress [330]. Along with PECAM-1 and VE-cadherin, KDR was shown to be essential for flow-induced EC alignment which is regulated by conformational activation of integrins. Specifically, flow-induced phosphorylation of KDR was found necessary for activation of phosphatidylinositol-3-OH kinase (PI(3)K), a known activator of integrins. However, formation of new integrin-ECM bonds is also implicated in the flow-induced stimulation of nuclear factor-κB (NF-κB) [331], a transcription factor that regulates many pro-inflammatory genes associated with atherosclerosis. ECs exposed to low and disturbed flow patterns *in vitro*, such as those of atheroprone areas of the vasculature, have shown prolonged elevation of NF-κB activity whereas those exposed to high laminar flow had an initial increase followed by sustained lower activity levels [332]. It has thus been suggested that under sustained high shear stress other flow-induced mechanisms dominate and maintain the ECs in their atheroprotective state. For example, cytoskeletal changes associated with elongation may promote a non-inflammatory phenotype through upregulation of the transcription factor krüppel-like factor 2 [333]. Additionally, one of the aforementioned products of VEGF-KDR signaling, NO, is reportedly a potent inhibitor of NF-κB activity [334]. Interestingly, PI(3)K in conjunction with Akt activates eNOS and KDR also promotes this pathway under arterial levels of laminar shear stress independent of

VEGF [335]. These distinct ligand-independent roles in addition to its ligand-dependent effects highlight the importance of KDR in healthy ECs.

For purposes of identifying EPCs, KDR is generally used in tandem with other markers such as CD34, CD133, and CD14. While the effect of the presence or absence of KDR on selected cells has rarely been the subject of EPC studies, gathered evidence points to it as essential for positive outcomes associated with EPCs. Indirect evidence is provided by the fact that the KDR ligand VEGF is the one ubiquitous factor used for inducing *in vitro* endothelial differentiation, regardless of the markers used for progenitor cell selection. VEGF has been reported essential for the *in vitro* endothelial differentiation of $CD34^+$ progenitor cells derived from peripheral blood, bone marrow, umbilical cord blood, and fetal livers and is presumed to promote this change through KDR [336]. Other direct evidence has been provided by studies investigating the engraftment of KDR^+ peripheral blood cells into injured vasculature. In mice with femoral artery injuries, human $KDR^+/CD14^+$ cells were shown to contribute to reendothelialization without increased neointimal formation while this was not observed when KDR^- cells were used [89]. Additionally, the original study identifying circulating EPCs showed engraftment of mouse PBMNCs selected for Flk-1 into the endothelium of vasculature of ischemic tissue [78]. More recently, a subpopulation of stem cell-like bone marrow-derived $CD14^+$ cells also described as $CD34^{low}$ were reported to be a source capable of endothelial differentiation *in vitro* [85]. Interestingly, that study also found that 80% of circulating KDR^+ cells were $CD14^+/CD34^{low}$. So while there may exist populations of KDR^+ peripheral blood cells that would not be sources of EPCs, the cumulative evidence to date suggests that such a population is but a small subset of circulating KDR^+ cells.

3.3.3 Technical Considerations for Cell Capture from Flow

Arrest of flowing cells such as leukocytes and bone marrow-derived progenitor cells is a natural activity in the body at sites of vascular injury. This is a multistep process in which the initial tethering of cells via selectins and selectin ligands transitions to rolling and finally firm adhesion mediated by integrins and their ligands [337, 338]. Selectin-ligand interactions mediate tethering and rolling through rapid association and dissociation and this behavior depends on the high tensile strength of the bonds and ample cytoskeletal support of both the selectins and their ligands [339-342]. The nature of high-affinity antibody-antigen interactions presents different binding kinetics than cell

adhesion molecules and in the case of capture onto stents and grafts these interactions are counted on to mediate the firm arrest of desired cells from flow absent any complementary interactions (i.e. tethering and rolling). This type of abrupt arrest of nonrolling cells from fluid flow, termed bimodal adhesion, has been described previously for cells displaying receptor-bound antibodies passing over immobilized antigen [343]. Arrest of cells in this manner was strongly dependent on shear rate and the forward rate constant of bond formation. For antibody-coated capture surfaces, antibodies of insufficient affinity or bond formation kinetics or antigens lacking adequate cytoskeletal support may not be suitable.

Assuming that arrest of cells via receptor molecules is possible another important consideration for antibody-mediated capture is the conformation of the immobilized antibodies. The simplest form of protein immobilization, physical adsorption, results in denaturation and a variety of random conformations of the protein on the surface. Given the low number of binding sites available after passive adsorption of antibodies [344], reportedly as low as 3% for monoclonal antibodies adsorbed on polystyrene, a specific method for immobilizing antibodies with the fragment antigen binding (Fab) domains directed away from the surface would likely substantially improve capture efficiency. In addition to reducing the availability of Fab domains and the binding affinity, nonspecific immobilization makes available at least some fraction of the fragment crystallizable (Fc) chains. This could be troublesome for specific antibody-mediated capture from blood as receptors for Fc chains exist on many cells of the vasculature including ECs, platelets, and neutrophils [345-347]. Thus, the benefit of controlled immobilization is two-fold – increased availability of binding sites and decreased exposure of Fc chains. Attempts to increase sensitivity of protein microarrays and immunosensors have highlighted some means of achieving such immobilization. For example, an increase of analyte-binding capacity has been observed for antibodies biotinylated on their Fc chains and immobilized on streptavidin-coated surfaces [348]. An alternative method that does not require chemical modification of the antibody is to coat surfaces with protein G prior to coating with antibodies. Because protein G specifically binds the Fc region of immunoglobulin G (IgG) [349, 350], protein G-coated surfaces can be used to immobilize IgGs with the Fab domains directed away from the surface, a function others have exploited for immunosensor design [351]. Unfortunately, the method of antibody

immobilization in previous cell capture studies is not disclosed and so the effects of different immobilization strategies on capture selectivity and efficiency are not known.

3.3.4 Summary

Blood-contacting vascular prosthetics engineered to stimulate endothelialization via capture of EPCs is a promising idea that has returned mixed results. There are many possible reasons for this including the choice of CD34 as a target molecule and the specificity or lack thereof of capture interactions. While there are a number of marker molecules that are frequently used to select EPCs, KDR is unique among these markers in that it has a significant role in EC functions and in EPC differentiation. Additionally, the benefits of an early localized increase in KDR expression following implant-induced damage has recently been shown in pigs receiving coronary stents. Following stent implantation, KDR-transfected coronary artery segments showed early increases in KDR expression compared to untransfected controls and animals with transfected segments exhibited reduced neointimal hyperplasia and luminal narrowing at four weeks even though KDR expression had returned to basal levels by this time [352]. With reportedly less than 1% of peripheral blood $CD34^+$ cells being KDR^+ and vice versa [81, 85] targeting $CD34^+$ cells for capture certainly brings in very few cells expressing this important receptor and this may be one reason for the lack of protection provided by anti-CD34-coated grafts. Thus, a method for the efficient and specific capture of KDR^+ cells from flow is an alternative approach well worth investigating.

3.4 Materials and Methods

3.4.1 Surface Treatment

Before flow experiments, glass coverslips were coated with 50 μg/ml monoclonal mouse anti-human KDR IgG1 (anti-KDR mAb, R&D Systems, Minneapolis, MN) or 50 μg/ml monoclonal mouse anti-human TGF-β1 IgG1 (anti-TGFβ1 mAb, PeproTech, Rocky Hill, NJ) for 1 hour. Coverslips were then blocked with 5 mg/ml heat-denatured bovine serum albumin (BSA, Sigma, St. Louis, MO) for 30 minutes. Coverslips coated with BSA only were used as controls. To evaluate specificity, anti-KDR mAb-coated slides were incubated with 25 μg/ml recombinant human KDR/Fc chimera (rhKDR, Sigma) prior to flow. The ability to improve capture by antibody orientation was tested by

first adsorbing 125 μg/ml recombinant protein G (BioVision, Mountain View, CA) followed by BSA and then 50 μg/ml anti-KDR mAb. Coverslips coated with protein G and BSA only were used as controls in this case. This preparation for antibody orientation was also used in subsequent experiments to test the selectivity when HUVECs were mixed with baboon smooth muscle cells (BaSMCs).

3.4.2 Surface Characterization

The surface densities of anti-KDR mAb on bare glass and protein G-modified glass were quantified using ^{125}I-anti-KDR and a Wizard™ 3" 1480 Automatic Gamma Counter (Perkin Elmer, Waltham, MA). Anti-KDR mAb was iodinated with Na^{125}I using IODO-BEADS® Iodination Reagent (Pierce, Rockford, IL) per the manufacturer's instructions. Trichloroacetic acid (10%) was used to determine the efficiency of antibody labeling after allowing 15 minutes of reaction time. Labeled anti-KDR mAb (^{125}I-anti-KDR mAb) was separated from free reagents by size-exclusion chromatography using BSA-blocked Sephadex® G-25 M PD-10 columns (GE Healthcare, Piscataway, NJ). Fractions were collected and radioactivity counted to determine ^{125}I-anti-KDR mAb-containing samples for pooling. Specific activity of ^{125}I-anti-KDR mAb was determined using the gamma counter and the labeled antibody was stored at 4°C.

To determine the surface density for anti-KDR mAb coating glass for 1 hour at room temperature, circular glass coverslips were prepared as described above and coated with ^{125}I-anti-KDR mAb mixed with unlabeled anti-KDR mAb diluted in Dulbecco's phosphate-buffered saline (PBS, Sigma) over a range of coating concentrations (12.5-75 μg/ml). After rinsing coverslips extensively with PBS, adsorbed ^{125}I-anti-KDR mAb was quantified with the gamma counter and radioactive counts (cpm) were converted to adsorbed anti-KDR mAb surface densities (μg/cm^2). To compare the surface density of adsorbed anti-KDR mAb to protein G-immobilized anti-KDR mAb, coverslips were prepared as described for flow experiments and coated with ^{125}I-anti-KDR mAb mixed with unlabeled anti-KDR mAb at the selected coating concentration (50 μg/ml). For both cases, the ratio of labeled to unlabeled anti-KDR mAb (specific activity) was varied to confirm that the iodination process did not hinder the ability of protein G to bind the antibody nor alter the adsorption behavior of anti-KDR mAb.

3.4.3 Cell Culture, Characterization, and Preparation

HUVECs (generously donated by Dr. Robert M. Nerem, Georgia Institute of Technology) were maintained in EC basal medium-2 (EBM-2, Cambrex BioScience Inc., Walkersville, MD) supplemented with EGM-2 SingleQuots® (Cambrex) and 10% fetal bovine serum (FBS, HyClone, Logan, UT). BaSMCs were isolated as described previously [353] and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% FBS (Gibco), 0.3 mg/mL L-glutamine (Gibco), and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL; amphotericin B, 0.25 μg/mL; Gibco). All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

The presence or absence of KDR on the surfaces of HUVECs and BaSMCs was confirmed via immunofluorescent (IF) staining. Briefly, cells grown in collagen-coated chamber slides were rinsed with PBS and fixed with 3.2% formaldehyde for 10 min at room temperature (RT). Fixed cells were rinsed thoroughly with PBS and incubated with Image-iT FX Signal Enhancer (Molecular Probes, Eugene, OR) for 30 min in a humidified chamber to reduce background staining. Wells were then rinsed with PBS, blocked with 1% BSA for 30 min at RT, and then incubated with primary anti-KDR mAb (1:1500) for 45 min at RT. After multiple rinses with PBS, Alexa-488-conjugated secondary detector (1:1000; Molecular Probes) was added for 30 min at RT in the dark. Finally, wells were rinsed, counterstained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 5 min, rinsed again and mounted in mounting medium for visualization on an inverted microscope (Nikon).

On the day of flow studies, cells were detached by incubation with 0.2% ethylenediamine tetraacetic acid (EDTA) in PBS for 10 minutes at 37°C followed by 2-3 minutes with recombinant trypsin replacement (TrypLE™, Gibco). Cells were suspended in basal medium and until use stored at 4°C in well plates coated with BSA. In experiments with mixed cells, prior to detachment HUVECs and BaSMCs were treated with CellTracker™ Orange and Green (Cambrex), respectively, according to the manufacturer's protocol.

3.4.4 Flow Cytometry Analysis of Human PBMNCs

Blood from healthy volunteers was collected into acid citrate dextrose anticoagulant solution (1:10) and PBMNCs were isolated using centrifugation over Histopaque-1077 density gradient (Sigma). Briefly, blood was diluted 1:1 with Hanks'

Balanced Salt Solution (HBSS) and underlayed with 12 ml Histopaque-1077. Tubes were spun at 800×g for 30 min and the resulting mononuclear cell layer was isolated and washed three times with HBSS. Isolated PBMNCs were resuspended in PBS with 0.5% BSA, Fc-blocked with purified human immunoglobulin (Sigma) for 15 min at RT, and then stained with FITC-tagged anti-human KDR mAb (R&D Systems) at 4°C for 30 min. Stained cell suspensions were then rinsed with PBS containing 0.5% BSA before adding propidium iodide to exclude dead cells from analysis. Cells were kept cold until analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences). Unstained PBMNCs and those stained with propidium iodide only were used as negative controls and to establish the instrument settings.

3.4.5 Flow Chamber Configuration and Capture Studies

A circular parallel plate flow chamber (GlycoTech, Gaithersburg, MD) mounted on an inverted microscope (Zeiss, Thornwood, NY) and maintained at 37°C was used for flow studies (Fig. 3.1). The flow chamber consists of a cast acrylic flow deck (upper plate) with inlet and outlet ports, a silicon rubber gasket to control chamber size, and a vacuum port to hold the chamber together. The lower plate of the flow chamber for these experiments utilizes a circular glass coverslip on which antibodies were adsorbed. A sample syringe reservoir and a rinsing syringe reservoir are connected to the entrance port through a chemically inert solenoid Y valve (The Lee Company, Westbrook, CT). The exit port is connected to an infuse/withdraw syringe pump (Harvard Apparatus, Holliston, MA) to control flow rate through the chamber. The microscope is equipped with a CCD camera (Axiocam MRm, Zeiss) and imaging software (Stallion SlideBook v4.1.0.10) for monitoring cell arrest. For one-dimensional laminar flow, the shear rate (γ) is related to the volumetric flow rate (Q) by:

$$\gamma = \frac{6Q}{wh^2}$$

where w is the channel width and h is the channel height.

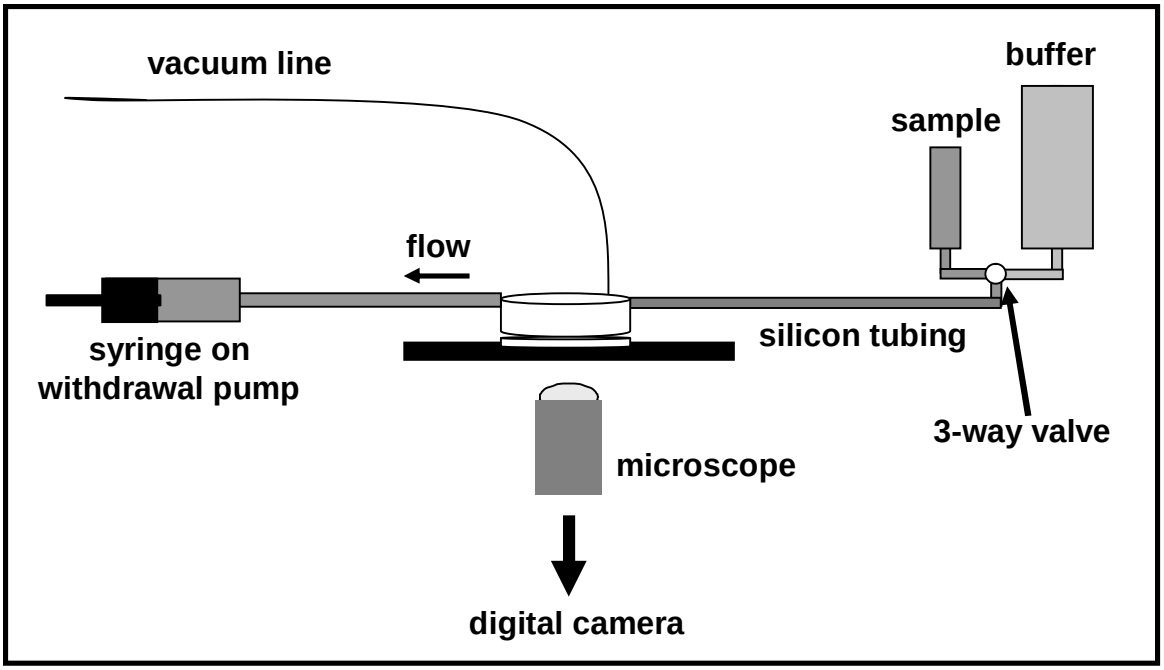

Figure 3.1. Flow chamber setup. Diagram of circular parallel plate flow chamber provided by and adapted from Dr. Owen McCarty.

At the time of flow experiments, cells were diluted to 8×10^5 or 1.5×10^6 total cells/ml in 0.4 ml serum-free medium and perfused through the flow chamber at a low shear rate (50 s^{-1}) over the surfaces described above. In experiments with mixed cells, HUVECs were 1, 10, or 100% of the total 1.5×10^6 cells/ml. At the conclusion of a single pass of flow, either the total number of cells in the flow chamber was counted or 20 fields of view (10× objective) were imaged and the number of attached cells counted. Manual counts confirmed that 20 images were sufficient to determine the number of attached cells per mm^2.

3.4.6 Statistical Analysis

SPSS 13.0 (SPSS Inc.) was used for statistical analysis. Student's t-tests and one-way analysis of variance (ANOVA) were used to assess statistical significance which was defined as $p<0.05$.

3.5 Results

3.5.1 Surface Characterization

To determine an optimal coating concentration of antibody in cell capture studies, anti-KDR mAb adsorption onto glass was measured as a function of anti-KDR mAb coating concentration using radiolabeled antibody. The results of anti-KDR mAb adsorption over a range of 12.5-75 μg/ml exhibited a typical adsorption profile (Fig. 3.2). Based on this curve, a coating concentration of 50 μg/ml was chosen for all cell capture studies. These results are similar to reported adsorption profiles of monoclonal antibodies on other materials [354].

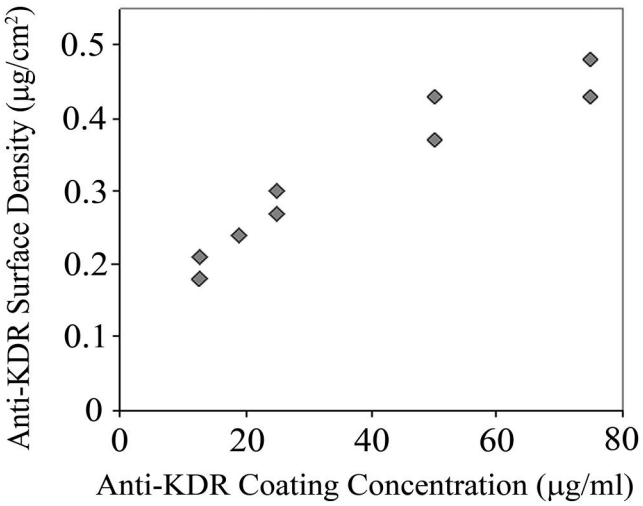

Figure 3.2. Anti-KDR mAb adsorption profile. Circular glass coverslips were coated with ^{125}I-anti-KDR mAb mixed with unlabeled anti-KDR mAb diluted in PBS at the indicated total concentrations. After 1 hour, coverslips were rinsed with PBS and adsorbed ^{125}I-anti-KDR mAb was quantified with a gamma counter. Radioactive counts (cpm) were converted to total adsorbed anti-KDR mAb surface densities (μg/cm^2) based on ratios of labeled to unlabeled mAb. Results are from two independent trials.

To compare the surface density of adsorbed anti-KDR mAb to protein G-immobilized anti-KDR mAb, 50 μg/ml radiolabeled anti-KDR mAb was adsorbed directly on coverslips or on BSA-blocked protein G-coated coverslips. In both cases the results were consistent using radiolabeled antibody with three different specific activities, indicating that the iodination procedure did not hinder the ability of protein G to bind the antibody nor alter the adsorption behavior of anti-KDR mAb. The surface density of total

anti-KDR mAb on bare glass was 0.42 ± 0.03 μg/cm^2 compared to 0.35 ± 0.03 μg/cm^2 on protein G-modified glass (means ± SD for N=4). At the same coating concentrations there was significantly less anti-KDR mAb on the surface when protein G was used to immobilize the antibody ($P<0.017$, independent samples t-test).

3.5.2 Capture Specificity and Optimization

To evaluate the role of cell Fc receptors in mediating EC arrest, we measured cell capture by adsorbed isotype-matched antibody to TGF-β1, a growth factor not embedded in the cell surface. Therefore, any arrest observed on such a surface could be attributed to Fc receptor interactions with the IgG1 Fc region. Cells were readily captured by anti-KDR mAb while arrest on anti-TGF-β1 mAb was almost nonexistent (Fig. 3.3). Cells flowing over anti-TGF-β1 mAb were rarely seen to arrest momentarily before detaching and moving on. This minimal nonspecific arrest indicates that the capture by anti-KDR mAb was not mediated by HUVEC Fc receptors. Furthermore, capture of HUVECs was blocked by adding rhKDR to the surface before flow, indicating that arrest is indeed mediated by antibody-antigen interactions (Fig. 3.4). Because rhKDR is an Fc chimera and thus adds more Fc fragments to the surface, this reduced capture also serves as further proof that the Fc region is not involved in cell arrest from flow. While anti-KDR mAb-mediated arrest was seen at 50 s^{-1}, we found that capture efficiency dramatically decreased at higher flow rates (~14-fold decrease at 100 s^{-1}). However, captured cells were stably adherent, as the shear rate was increased to >1000 s^{-1} after each experiment with minimal detachment of cells observed.

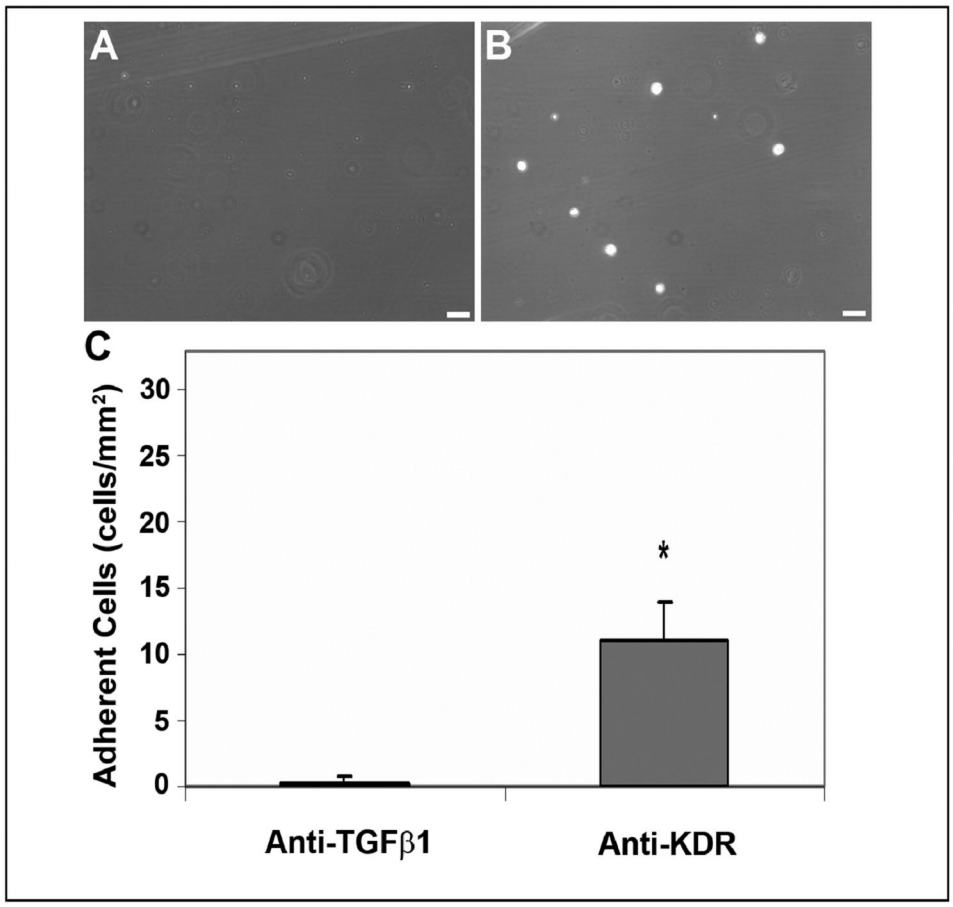

Figure 3.3. Capture of HUVECs from flow by anti-KDR mAb is not mediated by IgG1 Fc chain. Coverslips were coated with 50 μg/ml anti-KDR or anti-TGFβ1 mAbs followed by 5 mg/ml heat-denatured BSA prior to flow (8×10^5 HUVECs/ml, 50 s^{-1}). Representative images of average captured-cell density on anti-TGFβ1 (**A**) and anti-KDR (**B**). Scale bars=50 μm. (**C**) Values are mean number of cells/mm^2 ± SD for N=4 flow experiments. *P<0.004 compared to nonspecific arrest (independent samples t-test).

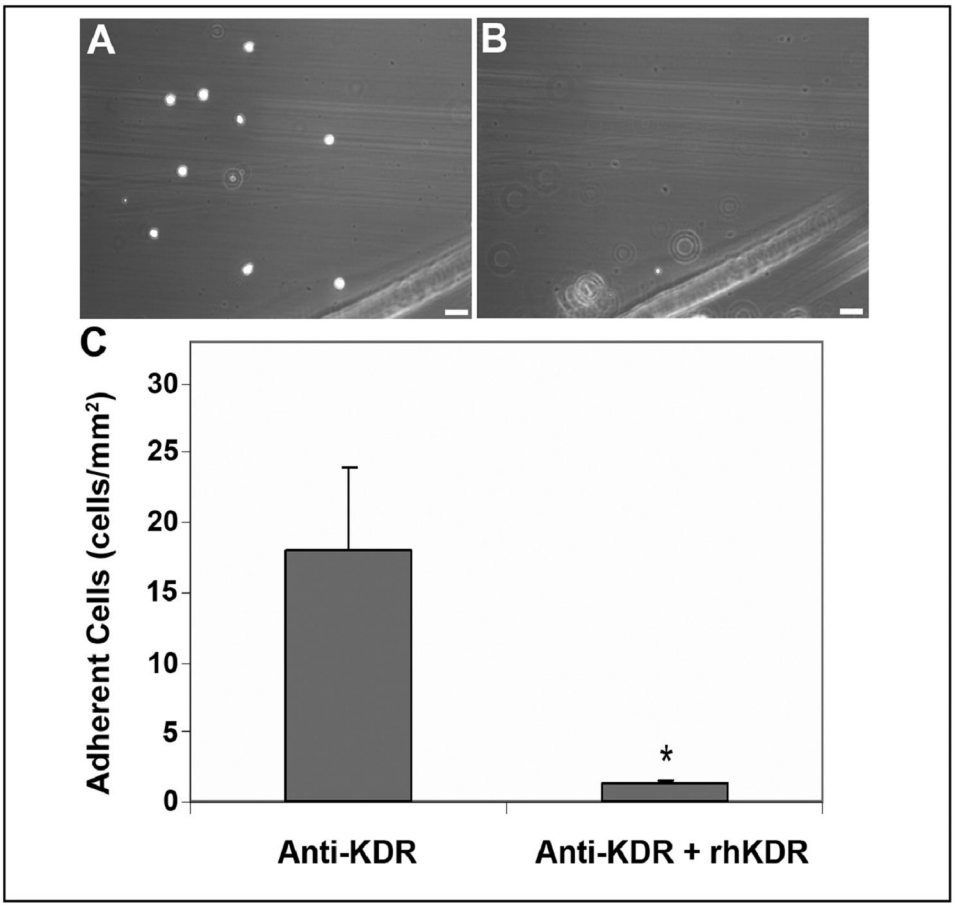

Figure 3.4. Capture of HUVECs from flow by anti-KDR mAb is blocked by rhKDR. Anti-KDR mAb-coated coverslips were incubated with 25 μg/ml rhKDR prior to flow (8×10^5 HUVECs/ml, 50 s^{-1}). Representative images of average captured-cell density on anti-KDR mAb (**A**) and anti-KDR mAb blocked with rhKDR (**B**). Scale bars=50 μm. (**C**) Values are mean number of cells/mm^2 ± SD for N=3 flow experiments. *P<0.039 compared to unblocked (independent samples t-test).

In order to increase capture efficiency, recombinant protein G was adsorbed prior to anti-KDR mAb. Protein G preferentially binds the Fc chain of antibodies and can thus increase availability of antigen-binding domains over randomly adsorbed antibody [349-351]. As shown in Fig. 3.5, protein G-orientation of the antibody increased the capture by approximately 2.5-fold despite a lower antibody density on the surface. Cell arrest on surfaces coated with protein G and BSA without antibody was the same as on BSA alone, that is only incidental. The lower total antibody density combined with the higher cell capture and lack of capture by protein G alone indicates that protein G effectively orients the antibody to increase cell capture.

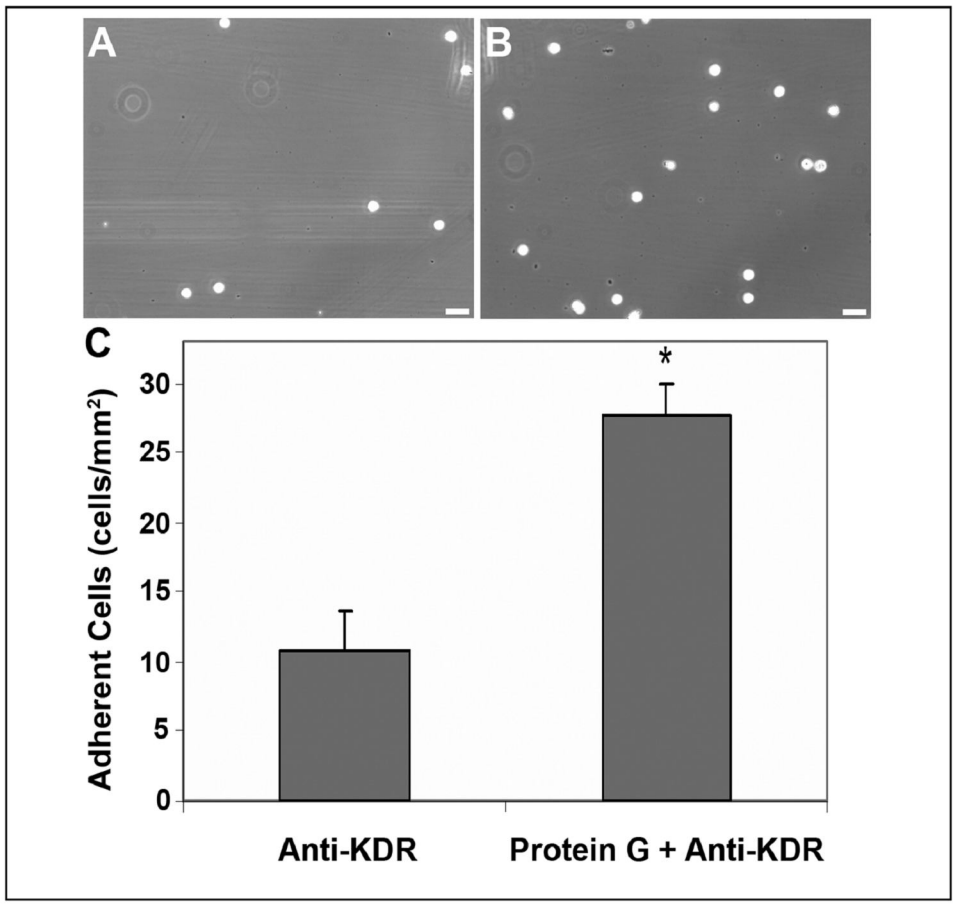

Figure 3.5. Protein G orients anti-KDR mAb for increased capture of HUVECs. Recombinant protein G (125 μg/ml) was first adsorbed followed by 5 mg/ml BSA and 50 μg/ml anti-KDR mAb prior to flow (8×10^5 HUVECs/ml, 50 s^{-1}). Representative images of average captured-cell density on anti-KDR (**A**) and protein G-immobilized anti-KDR (**B**). Scale bars=50 μm. (**C**) Values are mean number of cells/mm^2 ± SD for N=3 flow experiments. *P<0.001 compared to adsorbed (independent samples t-test).

3.5.3 Capture Selectivity

To evaluate the selectivity and efficiency of capture from a heterogeneous mixture of cells, we investigated the capture of HUVECs from flow when they are present in small numbers compared to cells that lack surface expression of KDR. Fig. 3.6 shows representative images of IF staining for KDR confirming the discrepancy in surface expression on HUVECs compared to BaSMCs. As we found no detectable surface expression of KDR in BaSMCs, these were used as a flowing population of cells without surface KDR. To establish relevant ratios of the two cell types to use in this model, we performed flow cytometry analysis with FITC-tagged anti-KDR mAb on

human PBMNCs. Fig. 3.7 shows a representative histogram from the five donors whose cells were analyzed. From these donors, we found 3.4 ± 1.3% KDR^+ cells, corresponding to ~5×10^4 – 1.5×10^5 KDR^+ cells/ml of whole blood.

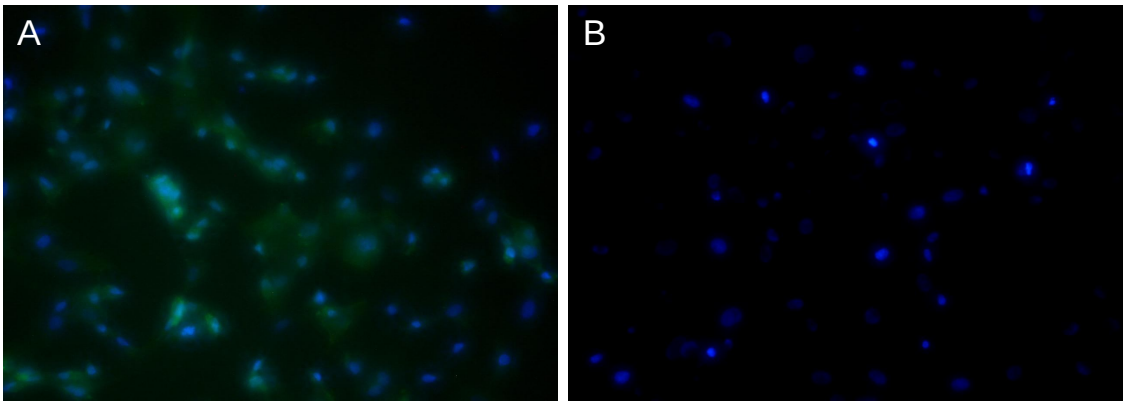

Figure 3.6. Representative IF staining for KDR on HUVECs and BaSMCs. Cultures of adherent HUVECs (**A**) and BaSMCs (**B**) in chamber slides were fixed and stained with primary anti-KDR mAb followed by Alexa-488-conjugated secondary detector. Negative controls where the primary antibody was omitted were used for background correction.

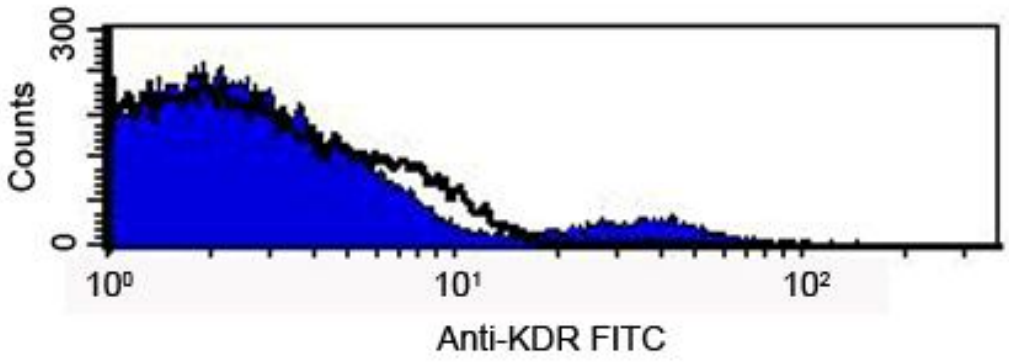

Figure 3.7. Representative flow cytometry histogram for human PBMNCs stained for KDR. Cells were Fc-blocked and stained with FITC-conjugated anti-human KDR mAb (blue-filled histogram) or with propidium iodide only (open histogram). The mean percentage of KDR^+ cells was 3.4 ± 1.3% of total PBMNCs for N=5 cytometry experiments.

For flow experiments with HUVEC/BaSMC mixtures we chose to analyze cell capture at HUVEC concentrations bracketing the range of KDR^+ cells found by flow cytometry. Fig. 3.8 shows the capture of HUVECs when they are 1, 10, and 100% (1.5×10^4, 1.5×10^5, and 1.5×10^6 HUVECs/ml) of the population of flowing cells. BaSMC

capture was negligible in each case (0.06 ± 0.07 and 0 ± 0 cells/mm^2 for 1 and 10% HUVECs, respectively). The data from the studies of mixed populations of HUVECs and BaSMCs was also analyzed to determine the correlation of cell capture to the number of flowing HUVECs and whether the presence of BaSMCs in flow affected the efficiency of HUVEC capture by anti-KDR mAb. As shown in Fig. 3.9A, the number of HUVECs captured is directly related to the number of HUVECs perfused through the chamber regardless of the relative number of BaSMCs. Despite the higher variability, Fig. 3.9B further illustrates that the presence of BaSMCs did not negatively affect the efficiency of antibody-mediated capture of HUVECs from flow.

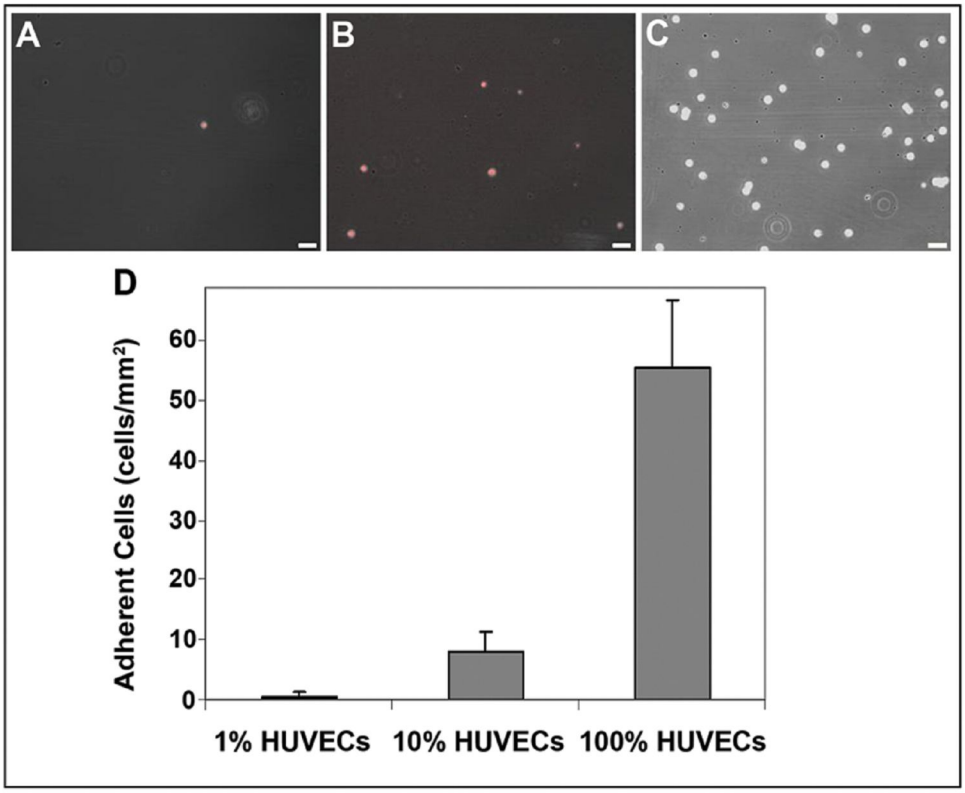

Figure 3.8. Selective capture of HUVECs mixed with BaSMCs by protein G-oriented anti-KDR mAb. Total cell concentration of 1.5×10^6 cells/ml perfused at 50 s^{-1}. Representative images of average captured-cell density with 1% (**A**), 10% (**B**), and 100% HUVECs (**C**). Images for 1 and 10% are overlays of phase and fluorescence. Scale bars=50 μm. (**D**) Values are mean number of cells/mm^2 ± SD for N=4 flow experiments. Mean BaSMCs captured were 0.06 ± 0.07 and 0 ± 0 cells/mm^2 for 1 and 10% HUVECs, respectively.

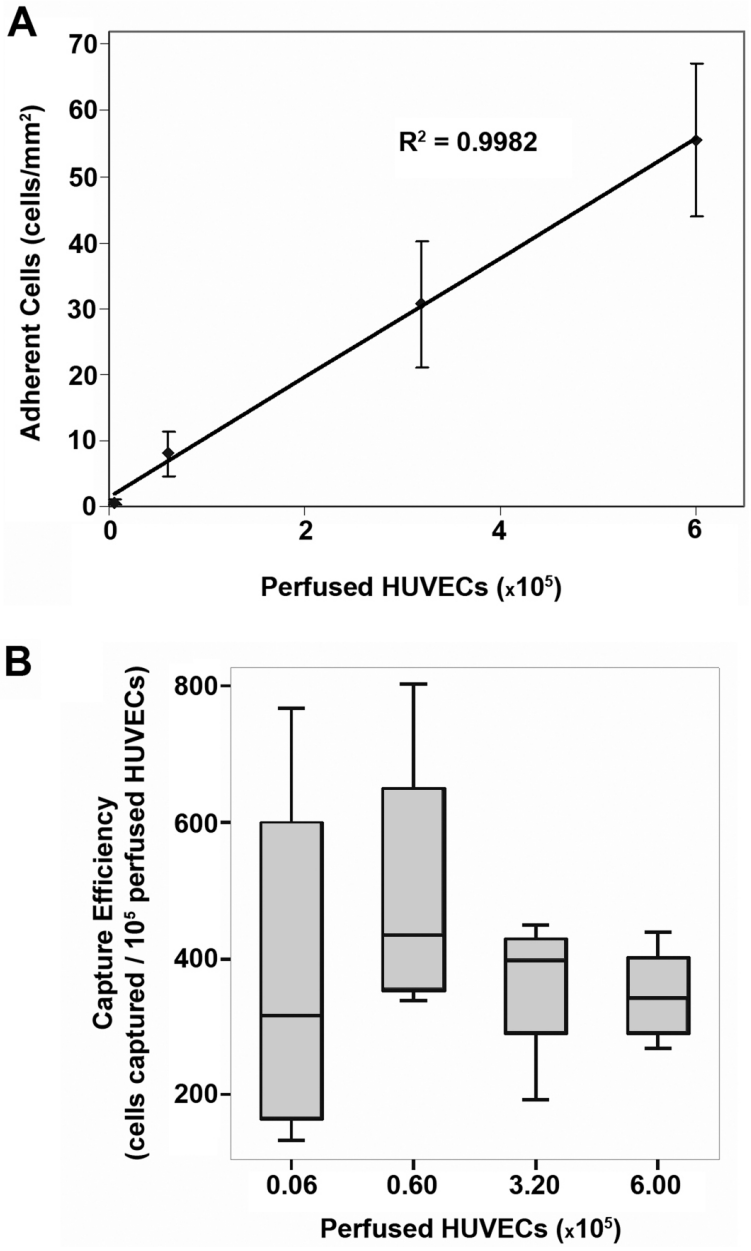

Figure 3.9. Relationship between number of HUVECs perfused over protein G-immobilized anti-KDR mAb and capture of cells in the presence or absence of BaSMCs. Total cell number is 6×10^5 for 0.06 and 0.60 (1% and 10% HUVECs); 3.20 and 6.00 are HUVECs only. (**A**) Linear correlation between capture (cells/mm^2 ± SD) and number of HUVECs perfused through flow chamber and (**B**) capture efficiency represented as number of cells captured per 10^5 perfused HUVECs for N=4 flow experiments. There was no significant difference for capture efficiency as assessed by ANOVA (P>0.05).

3.6 Discussion

In this study, we investigated whether cells expressing KDR could be captured from flow using surface-immobilized monoclonal anti-KDR antibody. KDR represents a possible target molecule for selection of EPCs from peripheral blood. While there is still considerable controversy over what constitutes the best marker for circulating cells that aid in reendothelialization *in vivo*, KDR is a leading candidate. Thus, KDR^+ PBMNCs represent an intriguing source of cells for endothelializing blood-contacting vascular implant devices.

Capture of cells with anti-CD34 has previously been demonstrated *in vivo* [130, 131], but CD34 is a ligand for selectins and thus may naturally be involved in tethering and arrest of cells expressing the molecule, albeit via different bond interactions [355, 356]. Normally, the arrest of cells from flow in the body at sites of vascular injury or inflammation is mediated by transient tethering interactions that cause rolling of the cells along the vessel wall before firm adhesion occurs [337, 338]. Tethering and rolling are due to rapid binding kinetics of selectins and their ligands, whereas firm adhesion is mediated by activation of integrins. Because KDR is a growth factor receptor that is structurally different from any of these adhesion molecules, we evaluated whether such a molecule could be used to arrest cells from flow via an antibody-antigen interaction. We show here that these interactions can indeed mediate cell capture of KDR^+ cells under low shear conditions. As expected there was no rolling phenomena observed and capture of cells was abrupt as has been described by others using an inverse system to ours (i.e. immobilized antigen, cell-bound antibody) [343]. Similar to our observations, they report a dramatic decrease in capture of cells at shear rates of 100 s^{-1} compared to 60 s^{-1} and lower; they also found negligible capture of cells via Fc receptors to immunoglobulin E at 60 s^{-1}. Another group has extensively characterized the antibody-mediated arrest of flowing lipid-based, gas-filled microbubbles that are conjugated with antibodies directed to adhesion molecules [357-360]. They have reported that microbubbles conjugated with anti-platelet selectin (P-selectin) mAb and those conjugated with anti-vascular cell adhesion molecule-1 (VCAM-1) mAb readily adhered on surfaces displaying the relevant antigens at low shear stresses (<0.7 dyn/cm^2) [357-359]. At higher shear stress, capture of microbubbles decreased substantially; however, microbubbles captured at low shear stress maintained adhesion remarkably well with increasing shear stresses presumably due to slow dissociation of the antigen-antibody

bonds [359]. If a viscosity of water at 37°C (0.6915 cP) is assumed for our study, the wall shear stress at which HUVECs were captured would be even lower (0.35 dyn/cm^2). Still, as was reported with microbubbles, we observed that following the short period of low shear rates in which cells were captured, antibody-mediated adhesion was not disrupted even at arterial levels of shear. While the shear rates we studied are well below arterial shear rates, the capture efficiencies are calculated for a single five-minute pass at continuous shear using the bulk cell concentration rather than the concentration that contacts the surface. Interestingly, microbubbles conjugated with anti-VCAM-1 mAb were observed to adhere when much higher shear stresses (12 dyn/cm^2) were briefly interrupted with drops in shear to create variations as occurs in pulsatile blood flow [357]. Furthermore, these microbubbles were able to adhere readily to VCAM-1-expressing aortic arches in mice, a location with very high peak shear stresses. It would be interesting then to see the effects of transient variations in shear on the capture of cells in our system. Although the antibody is immobilized and the antigen is cell-bound, it is likely that transient drops in shear would similarly increase our capture at high shear rates.

Given the expense associated with coating surfaces with antibodies, the most desirable surfaces for devices employing these tactics would be highly efficient with regard to antibody functionality. Because passive adsorption of antibodies results in low availability of antigen-binding domains, we evaluated the effect of the surface immobilization technique for the purposes of cell capture. Here, we investigated whether antibody orientation on the surface via an established method, adsorbed protein G, would provide significant improvement in capture efficiency over passive adsorption of antibody. Adsorbing protein G and blocking with BSA prior to applying antibodies to the surface resulted in significantly less antibody density on the surface. This phenomenon is consistent with ellipsometry observations of antibody surface density when physically adsorbed on silicon compared to immobilization via protein A-modified silicon [361]. Like protein G, protein A is a bacterial receptor known to specifically bind the Fc region of IgGs, albeit with lower affinity [349, 350]. Because the recombinant protein G had the albumin-binding domain removed, the lower antibody density may be limited by the amount of protein G on the surface or due to steric hindrance in this configuration. Despite lower antibody density on flow-contacting surfaces, protein G-orientation of anti-KDR mAb greatly increased the capture efficiency over directly adsorbed antibody. In

the aforementioned study using cell-bound antibodies and immobilized ligand, increasing ligand density increased adhesion at a low shear rate (68 s^{-1}) [343]. This was a modest increase compared to the effect of increasing the antibody density on the cell surface, because their immobilized ligand density was always greater than the cell-bound antibody density. Similarly, increasing the intercellular adhesion molecule-1 (ICAM-1) expression of adherent ECs increased the adhesion of anti-ICAM-1 mAb-conjugated microbubbles to the ECs but the effect of increasing anti-ICAM-1 density on the microbubbles was apparently much greater [360]. Additionally, increasing immobilized P-selectin surface density increased the adhesion of anti-P-selectin-conjugated microbubbles at low shear stress (0.3 dyn/cm^2), although the microbubble-bound anti-P-selectin density was not varied in this study [359]. Regardless of the relative effect of ligand density to that of cell/microbubble antibody density, each of these studies found an increase in capture when immobilized ligand density was increased. In contrast, the significant increase in HUVEC capture associated with a *decrease* in total surface density of antibody via protein G-immobilization in this study highlights the poor quality of adsorbed antibody for cell capture purposes.

Finally, we evaluated the selectivity and efficiency of capture from a heterogeneous mixture of cells using protein G-oriented anti-KDR mAb to capture HUVECs from flow when mixed with BaSMCs. This is important as KDR^+ cells are a small percentage of the PBMNC population. Using flow cytometry, we found KDR^+ cells to be ~2-5% of PBMNCs (~5×10^4 – 1.5×10^5 KDR^+ cells/ml of whole blood). Therefore, we assessed the capture of HUVECs when they are present in these amounts and less in the presence of BaSMCs. Anti-KDR mAb-mediated HUVEC capture was directly dependent on the number of flowing HUVECs and unaffected by large populations of cells lacking surface expression of KDR which did not arrest on the surfaces. This illustrates the feasibility of targeting this growth factor receptor to selectively capture cells from heterogeneous populations.

3.7 Limitations

HUVECs were used to study the ability of an antibody directed at KDR to arrest flowing cells onto a solid surface. HUVECs provide a good model cell type to evaluate such a platform as they express KDR on their surface. In contrast, BaSMCs are a good negative control due to their lack of KDR surface expression. However, both cell types

are substantially larger than circulating mononuclear cells which may affect the capture rate. Additionally, while we were able to control the relative percentage of KDR^+ cells by varying the number of HUVECs in a mixture with BaSMCs, we did not vary the expression levels of KDR. In a model of antibody-mediated capture inverse to ours – where antibodies were bound to the cells and the antigens to the substrate – others have found arrest from flow to be highly dependent on the number of cell-bound antibodies and on shear rate [343]. Specifically, the number of cell-bound antibodies had a major influence on capture near shear rates used in our study but made no difference at shear rates approaching 100 s^{-1}. Additionally, the adhesion of microbubbles conjugated with anti-ICAM-1 mAb to ECs was found to be dependent not only on the ICAM-1 expression level of ECs but also on the density of anti-ICAM-1 conjugated to microbubble surfaces [360]. KDR^+ PBMNCs may express a significantly different number of KDR molecules per cell surface area than HUVECs and the typical microvilliated topography of PBMNCs could also affect capture. To evaluate capture of KDR^+ PBMNCs, however, it will be necessary to devise methods for antibody immobilization different from the strategies described here for reasons discussed below.

While this study demonstrates that flowing KDR^+ cells can be captured via specific antibodies bound to solid surfaces and that orienting the antibodies increases the efficiency of capture, it should also be noted that such an orientation would likely be essential in blood-contacting surfaces. Our conclusion that Fc receptors were not involved in capture of HUVECs flowing over IgG-coated surfaces is in agreement with others' observations of poor Fc receptor-mediated arrest of basophilic leukemia cells via immobilized immunoglobulin E [343]. However, in a study where surface-adsorbed antibodies against EC membrane glycoproteins were used to promote adhesion, it was found that platelet aggregates formed on the surface when full-sized antibodies were adsorbed [354]. Conversely, minimal platelet reactivity was found for surface-adsorbed $F(ab')_2$ fragments, indicating that the Fc fragments were responsible for platelet activation. The implication of these domains for the observed aggregation is not surprising since platelets express an Fc receptor for immunoglobulin G [346]. Neutrophils also express Fc receptors that have recently been shown to be involved in capture and activation over immobilized immunoglobulin at up to 70 s^{-1}, although arrest was negligible at shear rates >140 s^{-1} [347]. Unfortunately, the described arrangement for orientation is likely not translatable to *in vivo* situations as we observed that purified

human PBMNCs bound with high frequency to surfaces coated with protein G only, even at shear rates greater than 50 s^{-1}. Therefore, even in the absence of platelets and red blood cells, the use of this immobilization strategy to evaluate the ability to select KDR^+ cells from purified PBMNCs is limited.

3.8 Conclusion

In this study, we determined that from a heterogeneous population of cells (HUVECs and BaSMCs), KDR^+ HUVECs could be selectively captured from flow by adsorbed anti-KDR mAb. Furthermore, cell capture was increased by immobilizing anti-KDR mAb with adsorbed protein G. Capture efficiency was not affected by varying either the number of HUVECs or BaSMCs perfused over the surface but rather by the method of antibody immobilization. KDR^+ cells from the peripheral blood represent a possible source of EPCs and we have shown here the ability to use this EPC marker to capture flowing cells onto a solid surface.

CHAPTER IV

Low Oxygen BM-MSC Chondrogenic Micropellets[2]

4.1 Abstract

Chondrogenesis of MSCs is typically induced when they are condensed into a single aggregate and exposed to TGF-β. Hypoxia, like aggregation and TGF-β delivery, may be crucial for complete chondrogenesis. However, the pellet dimensions and associated self-induced oxygen gradients of current chondrogenic methods may limit the effectiveness of *in vitro* differentiation and subsequent therapeutic uses. Here we describe the use of embryoid body-forming technology to produce microscopic aggregates of human BM-MSCs for chondrogenesis. These micropellet cultures (~170 cells/micropellet) as well as conventional pellet cultures (~2×10^5 cells/pellet) were chondrogenically induced under 20% and 2% oxygen environments for 14 days. Compared to conventional pellets under both environments, micropellets differentiated under 2% O_2 showed significantly increased sGAG production and more homogeneous distribution of proteoglycans and collagen II. Aggrecan and collagen II gene expression were increased in pellet cultures differentiated under 2% O_2 relative to 20% O_2 pellets but 2% O_2 micropellets showed even greater increases in these genes, as well as increased SOX9. These results suggest a more advanced stage of chondrogenesis in the micropellets accompanied by more homogeneous differentiation. Thus we present a new method for enhancing MSC chondrogenesis that reveals a unique relationship between oxygen tension and aggregate size. The inherent advantages of chondrogenic micropellets over a single macroscopic aggregate should allow for easy integration with a variety of cartilage engineering strategies.

[2] This research was originally published in *Cell Transplantation*. Markway, B.D., Tan, G.K., Brooke, G., Hudson, J.E., Cooper-White, J.J., and Doran, M.R., "Enhanced Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells in Low Oxygen Environment Micropellet Cultures," *Cell Transplant*. © 2010 by Cognizant Communications Corp.

4.2 Introduction

Articular cartilage has poor regenerative capacity following injury and degradation, due in part to its avascular nature. For some patients, ACI is a viable cartilage repair strategy, however this procedure requires the isolation of chondrocytes via a preliminary surgery which itself may result in further cartilage degeneration [147]. Additionally, the expansion of ACs can result in dedifferentiation and loss of the mechanical and phenotypic properties that make the cells ideal in the first place [144-146]. Adult MSCs may be an alternative autologous source for such applications due to their multipotency and relative ease of isolation and expansion [194]. The use of MSCs in cartilage repair, however, will be dependent on the development of efficient and controlled chondrogenesis methods. The most common current method used to induce scaffold-free chondrogenesis of MSCs *in vitro*, pellet culture, has long been used to study the mechanisms of differentiaton of these cells but is limited in its potential for integration with tissue engineering strategies. This is in part due to the frequent occurrence of uneven differentiation of MSCs and the associated heterogeneous synthesis of the major structural components of articular cartilage such as collagen II and the proteoglycan aggrecan. Mass transport limitations in macroscopic pellets may be at the heart of this problem but recent innovations in embryoid body-forming technology could provide a solution [362]. This technology designed to improve the homogeneity of embryoid body size has led to a commercial tissue culture product employing a microwell surface that makes possible the formation of thousands of individual microscopic cell aggregates. Another condition that may hinder outcomes of MSC chondrogenesis is the high oxygen tension of conventional tissue culture environments. The physiological environments of both articular cartilage and bone marrow are reported to exist within a range of 1 to 7% O_2 [363, 364] and the development of a low oxygen environment has been indicated as an important condition for chondrogenesis *in vivo* [264, 265]. Yet, most studies regarding the use of MSCs for cartilage regeneration perform expansion and chondrogenic differentiation under normoxic culture conditions.

The purpose of this research was to evaluate the effects of aggregate size on the *in vitro* chondrogenic differentiation of human BM-MSCs, and specifically to study the homogeneity and extent of this differentiaton compared to conventional macroscopic pellets. Additionally, oxygen tension was varied between the typical normoxic conditions

of cell culture and a hypoxic environment within the range of that found in both bone marrow and cartilage. Enhancing the *in vitro* chondrogenic differentiation of BM-MSCs has the potential to advance tissue engineering strategies such as ACI, and a culture platform that physically reduces mass transport limitations in a low oxygen environment was investigated for its potential to progress such aims.

4.3 Background

4.3.1 Pellet Culture Chondrogenesis of MSCs

In vitro chondrogenesis of human BM-MSCs in the presence of TGF-β1 was first described using high-density pellet cultures [202, 203]. Aggregate formation along with members of the TGF-β superfamily [202, 203, 313], may be essential for complete *in vitro* chondrogenesis of MSCs, at least in scaffold-free systems. As with chondrogenesis of mouse and chick embryonic mesenchymal cells, N-cadherin has been indicated as important for adult human MSC chondrogenesis *in vitro* [365]. Blocking of N-cadherin decreased proteoglycan production in pellet cultures of human trabecular bone-derived MSCs and, more specifically, significantly diminished TGF-β1-induced activation of the aggrecan promoter. Furthermore, N-cadherin expression was transiently increased early upon treatment with TGF-β1. Thus, pellet culture appears to help mimic the critical first stage of limb bud chondrogenesis by facilitating the close association of MSCs needed for cell-cell interactions.

While conventional pellet culture is an effective tool for studying MSC chondrogenesis, it is not without its limitations. The original studies describing chondrogenic differentiation of human BM-MSCs showed proteoglycan accumulation throughout pellets except the outer layers where the cells maintained a flattened, fibroblastic morphology [202, 203]. Collagen II deposition mirrored this distribution even as late as two to three weeks after chondrogenic induction was initiated. In the decade since these studies demonstrated the utility of pellet culture, the technique has become ubiquitous in the study of MSC chondrogenesis. Still, typical histological analyses of these macroscopic pellets consistently reveal heterogeneous staining of the chondrogenic-specific matrix [202, 203, 313, 314, 316, 317, 322-324]. Considering multiple studies of human BM-MSCs, two general patterns of collagen II and proteoglycan staining are often observed – one in which the periphery of the pellet lacks

these markers [202, 203, 314, 324] and another in which the central region of the pellet shows diminished staining [313, 314, 316, 317, 322, 323]. Table 4.1 summarizes the histological observations from a number of studies that used human BM-MSCs in pellet cultures. Because there are multiple non-trivial differences between the studies including source of bone marrow, isolation techniques, expansion procedures, chondrogenic medium composition, and pellet size, it is not possible to determine causative agents in the different patterns noted.

While heterogeneity is often noted, most BM-MSC chondrogenesis studies are designed to explore the effects of a particular factor on differentiation in the context of this model system and are not concerned with exploring the matrix distribution phenomenon. However, for cartilage engineering uses, developing an efficient differentiation methodology capable of producing homogeneous replacement tissue is of keen interest. Utilizing scaffolds is a popular tactic in cartilage engineering but they necessarily reduce the amount of cell-cell contact by filling space with the structural material. An alternative approach to this challenge is to take the principles of pellet culture and modify the physical parameters to generate scaffold-free neocartilage. For example, one factor to consider is the fluctuating mass transport properties of the increasingly dense pellet. With macroscopic pellets, the accumulation of collagen fibers, proteoglycans, and other matrix components undoubtedly alters the diffusive properties of the tissue over the weeks required for chondrogenesis. Murdoch et al recently reported that a more homogeneously distributed matrix could be obtained by inducing chondrogenic differentiation of human BM-MSCs in thin discs of cells in Transwell plates [317]. In this study, the uniformly short diffusional distances from both sides of the Transwell plate are proposed to improve the distribution of matrix accumulation in these discs. Furthermore, the authors speculate that substantial changes in tensile forces due to peripheral fiber accumulation in the large spherical masses of pellet cultures promote a fibrocartilaginous matrix whereas the lack of such changes in their flat discs favor a more cartilage-like matrix. This study provides evidence that the geometric parameters of chondrogenically induced BM-MSC cultures have a significant effect on the spatial pattern of differentiation. Thus, modifying the scaffold-free pellet culture system to minimize transport limitations is a potential avenue to improve homogeneity of differentiation, an essential outcome for downstream therapeutic applications.

Table 4.1. Heterogeneity of human BM-MSC pellet culture chondrogenesis.

Cells/Pellet	TGF-β Isoform	Histological Notes	Reference
2×10^5	10 ng/ml TGF-β1	14 days: collagen I mostly peripheral; collagen II and X only inside	[202]
2.5×10^5	10 ng/ml TGF-β3	14 days: collagen II in center, not at periphery 21 days: collagen II and aggrecan in center, not at periphery; collagen I at periphery only	[203]
2×10^5	10 ng/ml TGF-β1	14 days: light central staining for collagen II 21 days: more intense central staining for collagen II, not at periphery	[314]
2×10^5	10 ng/ml TGF-β2	14 days: zonal staining for collagen II (between periphery and center) 21 days: lighter but more uniform staining for collagen II	[314]
2×10^5	10 ng/ml TGF-β3	14 days: collagen II staining throughout except periphery 21 days: collagen II appears more zonal	[314]
2.5×10^5	10 ng/ml TGF-β3	14 days: collagen II and Alcian blue localized to border zone, absent in center	[313]
5×10^5	10 ng/ml TGF-β3	14 days: collagen II heterogeneous and light in central region; collagen I intense at surface and towards the center; collagen X zonal between periphery and center; aggrecan uniform	[317]
2×10^5	10 ng/ml TGF-β3	4 weeks: collagen II most intense at periphery, not in center	[316]
2.5×10^5	10 ng/ml TGF-β3	3 weeks: collagen II mostly at periphery; non-uniform aggrecan distribution	[322]
5×10^5	100 ng/ml TGF-β3	14 days: collagen II inside, not at periphery	[324]

4.3.2 Oxygen Tension and Chondrogenesis

Another critical factor in chondrogenic differentiation, and possibly in BM-MSC maintenance in general is oxygen tension. In the avascular developing limb bud a hypoxic environment is established and guides proper chondrogenesis and joint formation [264]. Degradation of the alpha subunit of the transcription factor HIF-1 (HIF-1α) is mediated by proline hydroxylation which requires molecular oxygen [366].

Consequently, in hypoxic environments HIF-1α is stable and able to exert its activity on hypoxia responsive element (HRE) sequences of target genes. Using conditional knockout of HIF-1α in mice, Provot et al recently found that this transcription factor was not necessary for condensation to occur but that chondrogenesis and later joint specification were substantially delayed without it [264]. They suggest that the condensing mesenchymal cells and the thickening perichondrium around the interzone of future joint sites promote a hypoxic environment conducive to HIF-1α stability and activity. Additionally, mRNA expression of the essential chondrogenic transcription factor SOX9 was reportedly not affected by hypoxia and the authors propose that insufficient expression of the HIF-1α target prolyl-4-hydroxylase α (I) (P4haI), which regulates posttranslational modifications of collagens, may be responsible for delays. The chondrogenic promoting effects of HIF-1α independent of SOX9 in murine mesenchymal cells is in agreement with the findings of another study, which also found hypoxia-mediated decreases in APase activity, mineralization, and expression of genes associated with hypertrophy [367]. Amarilio et al, however, reported that HIF-1α binds to the mouse SOX9 promoter and that its regulation of SOX9 is required for the onset of chondrogenesis in mouse limb buds [265]. Likewise, others have reported that hypoxia-induced chondrogenic changes of murine bone marrow stromal cells *in vitro* are mediated through increased SOX9 expression and specifically by HRE sequences in the SOX9 promoter [368]. Regardless of the mechanisms, the cumulative evidence points to hypoxia as a powerful positive regulatory force in developmental chondrogenesis.

Studies evaluating the effects of hypoxia on adult human ACs have similarly revealed elevated expression of chondrogenic genes *in vitro*. In human ACs, expression of SOX9 was upregulated in hypoxic conditions, resulting in increased expression of collagen II and aggrecan [369]. HIF-2α, as opposed to HIF-1α, was reported to be essential for these effects although a more recent study found that HIF-1α bound to the promoters of human genes for both SOX9 and aggrecan [370]. This study also found that HIF-1α inhibited selected genes for collagens generally characteristic of fibrous tissue and fibrocartilage – collagen I and collagen III. Paralleling the complexity reported for murine mesenchymal cells, hypoxia has also been found to promote the chondrocyte phenotype in human ACs through SOX9-independent gene regulation [371]. The results of these studies implicate hypoxia as a potent regulator of AC redifferentiation in addition

to its role in chondrogenesis and point to oxygen tension as an important parameter for the *in vitro* promotion and maintenance of the chondrocyte phenotype.

In recent years, a number of studies have shown the benefits of low oxygen tension with regards to BM-MSC culture and differentiation. Culture under low oxygen environment has been shown to increase expansion potential of BM-MSCs [372-376]. Furthermore, post-hypoxia exposure differentiation studies have shown these cells to maintain multilineage differentiation capacity with enhanced chondrogenic potential [376, 377] and variable adipogenic and osteogenic potential [372-374, 377, 378]. The few studies utilizing low oxygen *during* chondrogenic differentiation also indicate improved outcomes. Although adipogenic and osteogenic differentiation of human MSCs under 3% O_2 was reduced [372], human Ad-MSCs in both alginate gels and aggregate culture under hypoxic environments showed increased chondrogenesis [364, 379]. Likewise, low oxygen tension enhanced chondrogenic differentiation of high-density cultures of bovine, mouse, and rat BM-MSCs [368, 380, 381]. Still, there is void of information regarding the effects of oxygen tension during chondrogenesis of human BM-MSCs.

4.3.3 Summary

BM-MSCs are a promising source of cells for tissue engineering neocartilage, whether used as a cellular input to ACI or in combination with novel scaffolds. However, the successful use of MSCs will depend on the engineering of biomaterials capable of promoting robust chondrogenesis or, alternatively, the development of scaffold-free methods that allow for finely controlled chondrogenesis and that are compatible with current tissue engineering strategies. Given the importance of cell-cell contact in chondrogenesis, creating small cellular aggregates in chondrogenic induction medium is one potential approach. Recently, Ungrin et al developed a microfabrication-based non-adhesive surface for the culture of thousands of individual aggregates of embryonic stem cells to improve embryoid body homogeneity and differentiation [362] . Such methods applied to MSC chondrogenesis could provide essential cell-cell contact signals, overcome presumed diffusional limits of current well-established protocols, and provide a cellular output compatible with many cartilage engineering techniques. However, there is still much to learn about the signals that drive chondrogenesis and particularly the formation of permanent articular cartilage so that appropriate agents can be implemented in chondrogenic induction of MSCs. One important environmental cue in

developmental chondrogenesis, hypoxia, is also potentially a critical signal for the formation of permanent articular cartilage [264]. Based on promising evidence gathered from hypoxia-exposed chondrogenesis of MSCs from other species and tissues, a low oxygen environment applied to human BM-MSCs likely enhances chondrogenesis of these cells. The combination of this environmental factor with the formation of small MSC aggregates then has potential to substantially improve the generation of neocartilage for use in tissue engineering.

4.4 Materials and Methods

4.4.1 Human BM-MSC Isolation and Culture

Full informed patient consent was obtained in all cases and ethical approval granted through the Mater Health Services Human Research Ethics Committee in accordance with the Australian National Health and Medical Research Council's Statement on Ethical Conduct in Research Involving Humans. Approximately 10 ml bone marrow was taken from iliac crest of healthy donors. The sample was diluted 1:1 with PBS and underlayed with 12 ml Ficoll-Paque Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Tubes were spun at 535×g for 20 min. Interface cells were washed and resuspended in low glucose Dulbecco's modified Eagle's medium (DMEM-LG; Gibco Life Technologies, Grand Island, NY) supplemented with 20% FBS (Gibco) and 50 μg/ml gentamicin (Amersham Pharmacia Biotech, Uppsala, Sweden) and placed in tissue culture flasks. After 48 hours, non-adherent cells were removed by washing with PBS and remaining adherent cells further cultured with medium changes every 3-4 days. Cells generally approached confluence after 14-20 days and were then passaged and expanded. After the second passage, cells were immunophenotyped by flow cytometry (monoclonal antibodies from BD Biosciences Pharmingen, San Diego, CA) and were functionally assessed for differentiation potential. Cells were deemed MSCs if they were $CD45^-$, $CD73^+$, $CD90^+$, $CD105^+$ and showed adipo-, osteo- and chondrogenic differentiation potential as described previously [382].

For these experiments, second passage BM-MSCs were expanded in DMEM-LG supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (1% PS; Gibco) in an incubator with a 2% O_2 atmosphere due to the aforementioned evidence of

the benefits of hypoxic preconditioning on MSC chondrogenesis. BM-MSCs from three different donors at fourth passage were used in chondrogenic assays.

4.4.2 Chondrogenic Differentiation

BM-MSCs were differentiated as conventional pellet cultures in 15-ml polypropylene tubes or micropellet cultures formed in AggreWell™ 400 plates (STEMCELL Technologies, Vancouver, BC, Canada). BM-MSCs were grown to near confluence, detached using recombinant trypsin replacement (TrypLE™; Gibco), and placed in serum-free chondrogenic induction medium consisting of high glucose DMEM (DMEM-HG; Gibco) containing 10 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ), 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 200 µM ascorbic acid 2-phosphate (Sigma), 100 µg/ml sodium pyruvate (Sigma), 40 µg/ml proline (Sigma), 1× ITS+ (Gibco), and 1% PS. Pellet or micropellet cultures (Fig. 4.1) were formed by centrifuging 2×10^5 cells at 500×g in chondrogenic induction medium and then culturing in a 2% O_2 or 20% O_2 atmosphere for a further 14 days. In this study, we used TGF-β1 which is known to induce chondrogenic differentiation of MSCs but possibly at a lesser rate than TGF-β3 [314]. This allowed us to evaluate micropellet differentiation at a time point (14 days) where chondrogenesis would be initiated in conventional pellet cultures but still at an early stage [236].

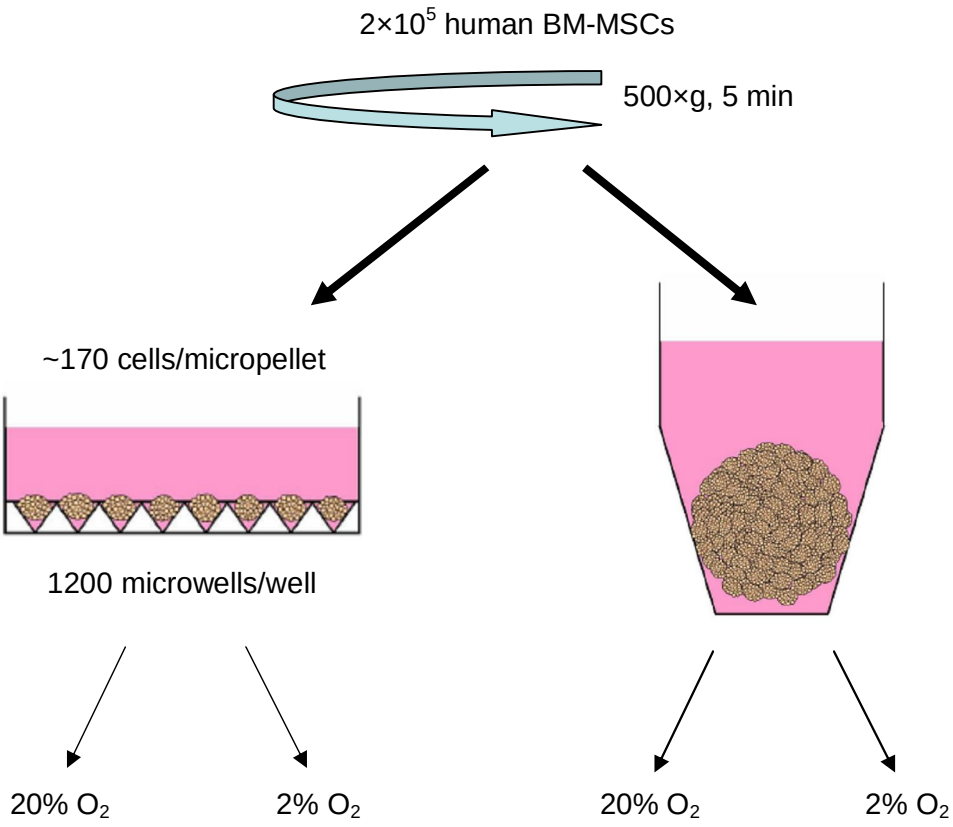

Figure 4.1. Diagram of micropellet and pellet culture platforms for BM-MSC chondrogenic induction. Human BM-MSCs were centrifuged in either Aggrewell™ 400 plates or 15-ml polypropylene tubes and cultured in chondrogenic induction medium under 20% or 2% O_2 atmosphere for 14 days. Medium was changed every 3-4 days.

4.4.3 Sulfated Glycosaminoglycan Quantification

Medium from pellet and micropellet cultures was collected and stored at -80°C at each medium replacement, every 3 to 4 days. At the end of 14 days, micropellets were centrifuged to a single pellet. Micropellets and pellets were digested with 1.6 U/ml papain (Sigma) in buffer solution (0.1 M sodium acetate, 5 mM EDTA disodium, 5 mM L-cysteine; pH 6.5) at 60°C overnight. The sGAG content and DNA were quantified with 1,9-dimethymethylene blue (DMB; Sigma) and Hoechst 33342 (Molecular Probes, Eugene, OR) as described in detail by Liebman and Goldberg [383]. Additionally, the DMB assay was used to quantify sGAGs released into the medium collected at days 3, 6, 10, and 14. Briefly, digest or supernatant samples were diluted in 1% BSA in PBS and diluted samples added to clear 96-well plates. Equal volume of 2× DMB in formate buffer was added to each sample and standard, the plate shaken, and the absorbance at 525

nm read in a plate reader. To quantify DNA in papain digests, 2 µg/ml Hoechst 33342 solution was added to digest samples in black bottom 96-well plates and fluorescence was measured in a plate reader (excitation 355 nm / emission 455 nm). For sGAG and DNA quantification, shark chondroitin sulfate (Sigma) and calf thymus DNA (Sigma) were used as the respective standards.

4.4.4 Histology and Immunohistochemistry

At the end of 14 days, micropellets and pellets were fixed in 4% formaldehyde, embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and snap-frozen in liquid nitrogen. Samples were cryosectioned onto Superfrost Plus glass slides, air-dried and stored at -80°C until use. Before staining, the sections were rinsed in 70% ethanol and Tris-buffered saline (TBS; pH 7.5) to remove OCT compound. To detect proteoglycan deposition, sections were stained with 0.1% toluidine blue (ProSciTech, Thuringowa, QLD, Australia) in 1% NaCl solution (pH 2.3) for 3 min and rinsed with distilled water. Stained sections were then dehydrated through 95% and absolute ethanol, cleared in xylene, and mounted with Permount resinous mounting medium (ProSciTech). Organization of fibrillar collagen was detected by staining with 0.1% Picrosirius red F3B (ProSciTech) as previously described [384]. Briefly, rinsed sections were stained with hematoxylin, rinsed in tap water, stained with 0.1% Picrosirius red F3B for 1 hour, and washed in 0.5% acetic acid for 30 min. Stained sections were then dehydrated through 95% and absolute ethanol, cleared in histolene, and mounted with Permount resinous mounting medium before visualizing with an Olympus BX61 microscope equipped with polarizing filters.

Localizations of collagen I and collagen II were determined by double IF staining. Briefly, the sections were digested with 0.01% pepsin (Sigma) in 0.01 M HCl (pH 2) at 37°C for 10 min, followed by 0.1% hyaluronidase (Sigma) in PBS (pH 5) at RT for 30 min. After three washes with TBS, cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked for 30 min at RT in TBS containing 2% BSA and 2% normal goat serum. The sections were then stained with a 1:50 dilution of both polyclonal rabbit anti-collagen I (Cedarlane Labs, Burlington, ON, Canada) and monoclonal mouse anti-collagen II (Lab Vision, Fremont, CA) primary antibodies for 2 hours at RT. This was followed by incubation with a mixture of secondary antibodies containing Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse

IgG (both from Molecular Probes; 1:200 dilution) for 1 hour at RT. After each staining step, unbound antibodies were washed with TBS containing 0.2% Tween-20. Nuclei were counterstained with 1 µg/ml Hoechst 33342 for 5 min at RT. The sections were mounted in a mixture of glycerol and PBS (9:1 v/v) and examined with an Olympus BX61 fluorescence microscope. Negative controls without primary antibodies were used for background correction.

4.4.5 Relative Gene Expression Analysis

On day 14, RNA was collected from micropellets and mechanically disrupted conventional pellets using the RNEasy Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. RNA was also collected from day 0 monolayer BM-MSCs. RNA samples were treated with DNase I (0.1 U/µl final; Fermentas, Glen Burnie, MD) for 30 min at 37°C and then heat-inactivated at 65°C for 5 min in the presence of 2.5 mM EDTA. DNase I-treated RNA samples (50 ng) were reverse transcribed using SuperScript III RT and oligo$(dT)_{20}$ in the presence of RNaseOUT (all from Invitrogen) as per the manufacturer's instructions and stored at -80°C until analysis.

Real-time quantitative polymerase chain reaction (qPCR) was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The cycling parameters were 50°C for 2 min, 95°C for 2 min and then 95°C for 3 s and 60°C for 30 s for a total of 40 cycles. The primers used are shown in Table 4.2 and were all from previously published papers [385-388]. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method relative to the house-keeping gene cyclophilin A due to the instability of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in oxygen-dependent studies [389, 390]. Specificity of products was confirmed by melt curve analysis and 3% agarose gel electrophoresis.

Cyclophilin A was stable among day 14 differentiated BM-MSCs, but differed in monolayer BM-MSCs and thus could not be used to compare between days 0 and 14. GAPDH, however, was stable between 2% O_2 micropellets and monolayer BM-MSCs. Thus for some genes, we quantified the change in 2% O_2 micropellets compared to monolayer BM-MSCs and used this in conjunction with the change among conditions calculated using cyclophilin A to indirectly estimate the change in the conventional pellets from monolayer BM-MSCs. However, this was deemed to be more susceptible to error and was only used as an indication of the general magnitude and direction of

changes in pellets compared to undifferentiated BM-MSCs. Therefore all data shown and subjected to statistical analysis is compared to the current standard for BM-MSC chondrogenesis, 20% O_2 pellets, and with cyclophilin A as a reference gene.

Table 4.2. Primers used for qPCR analysis of chondrogenesis.

Gene	Primers	Amplicon size (bp)	Reference
Cyclophilin A	Forward: CTCGAATAAGTTTGACTTGTGTTT Reverse: CTAGGCATGGGAGGGAACA	164	[386]
GAPDH	Forward: ATGGGGAAGGTGAAGGTCG Reverse: TAAAAGCAGCCCTGGTGACC	119	[385]
SOX9	Forward: TTCCGCGACGTGGACAT Reverse: TCAAACTCGTTGACATCGAAGGT	77	[387]
Aggrecan	Forward: TCGAGGACAGCGAGGCC Reverse: TCGAGGGTGTAGCGTGTAGAGA	85	[385]
Collagen II (COL2A1)	Forward: GGCAATAGCAGGTTCACGTACA Reverse: CGATAACAGTCTTGCCCCACTT	79	[385]
Collagen I (COL1A1)	Forward: CAGCCGCTTCACCTACAGC Reverse: TTTTGTATTCAATCACTGTCTTGCC	83	[385]
Versican	Forward: TGGAATGATGTTCCCTGCAA Reverse: AAGGTCTTGGCATTTTCTACAACAG	98	[385]
Collagen X (COL10A1)	Forward: CAAGGCACCATCTCCAGGAA Reverse: AAAGGGTATTTGTGGCAGCATATT	70	[385]
Runx2/Cbfa1	Forward: GGAGTGGACGAGGCAAGAGTTT Reverse: AGCTTCTGTCTGTGCCTTCTGG	133	[388]
Osteocalcin	Forward: GAAGCCCAGCGGTGCA Reverse: CACTACCTCGCTGCCCTCC	70	[385]

4.4.6 Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL) was used for ANOVA with Tukey post-hoc tests to assess statistical significance, which was defined as $p<0.05$. For qPCR data, statistical analysis was conducted on the ΔCt values and the mean fold-increase and 95% confidence intervals are represented by $2^{-\Delta\Delta Ct}$ evaluated at the mean ΔΔCt, at the lower confidence limit of ΔΔCt, and at the upper confidence limit of ΔΔCt between conditions [391].

4.5 Results

4.5.1 Micropellet Development in AggreWell Plates

The feasibility of creating micropellets of BM-MSCs in the AggreWell plates was evaluated by varying the number of cells per well from 2×10^5 to 1×10^4. We found that consistent aggregates could be formed under both 20% and 2% O_2 using 2×10^5 cells/well, a common number of MSCs used in conventional pellet culture. At this density, aggregates formed under both oxygen environments within 14 days, albeit with different morphologies (Fig. 4.2). Micropellets under 2% O_2 appeared to be more loosely aggregated while those under 20% O_2 formed smaller compacted masses. Micropellets from both oxygen environments were easily collected for analysis at day 14 using only a pipette to dislodge them from microwells. All subsequent studies therefore used 2×10^5 cells per centrifuge tube or per well (~170 cells/microwell).

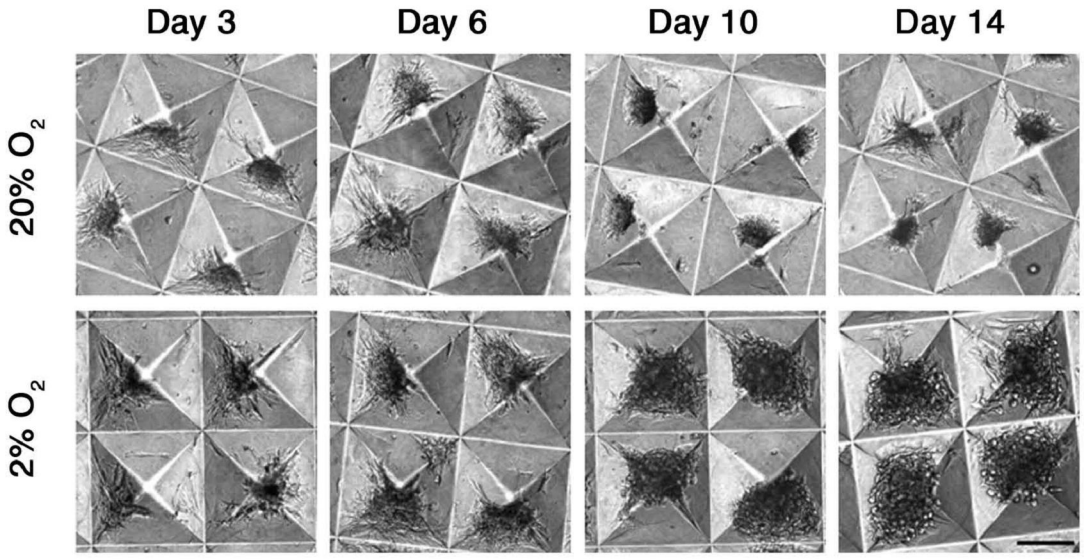

Figure 4.2. Morphological changes of human BM-MSC micropellets over 14 days of chondrogenic induction in AggreWell plates under 20% and 2% O_2 environments. Scale bar = 200 µm.

4.5.2 Proteoglycan Production

The proteoglycan production of micropellets was compared to that of conventional pellets by quantifying the total amount of sGAGs released over the course of chondrogenic induction and the amount retained within the aggregates' matrices. While 20% O_2 pellets displayed a relatively level profile, the amount released by 2% O_2 pellets showed a slightly increasing profile over 14 days (Fig. 4.3A). Micropellets differentiated at 20% O_2 generally displayed a steady but elevated release profile of sGAGs, while 2% O_2 micropellets had a distinctly linear sGAG release profile (Fig. 4.3B). The 2% O_2 pellets retained nearly twice the fraction of their total sGAGs as the 20% O_2 pellets (Fig. 4.3C) and correspondingly produced twice as much per µg of DNA within the matrix (Fig. 4.3D). Micropellets cultured at 2% O_2, meanwhile, produced 8.2- and 4.0-fold more total sGAGs than pellets at 20% and 2% O_2, respectively (Fig. 4.3C). The increase was also reflected in the amount of sGAGs retained with 6.3- and 1.6-fold more sGAGs within the aggregate mass of 2% O_2 micropellets. While 2% O_2 micropellets retained less than half the fraction of their total as 2% O_2 pellets (Fig. 4.3C), the increase in total released was of such a magnitude that the amount within the aggregates' matrices per DNA was not significantly different (Fig. 4.3D). The compact 20% O_2

micropellets were typically found to have very little DNA after 14 days and a low amount of sGAGs within the collected aggregate mass. We suspected that these micropellets experienced substantial cell death as there were often relatively few remaining after 10-14 days. Per DNA, the amount of sGAGs was statistically equivalent to that produced in the conventional 20% O_2 pellet (Fig. 4.3D).

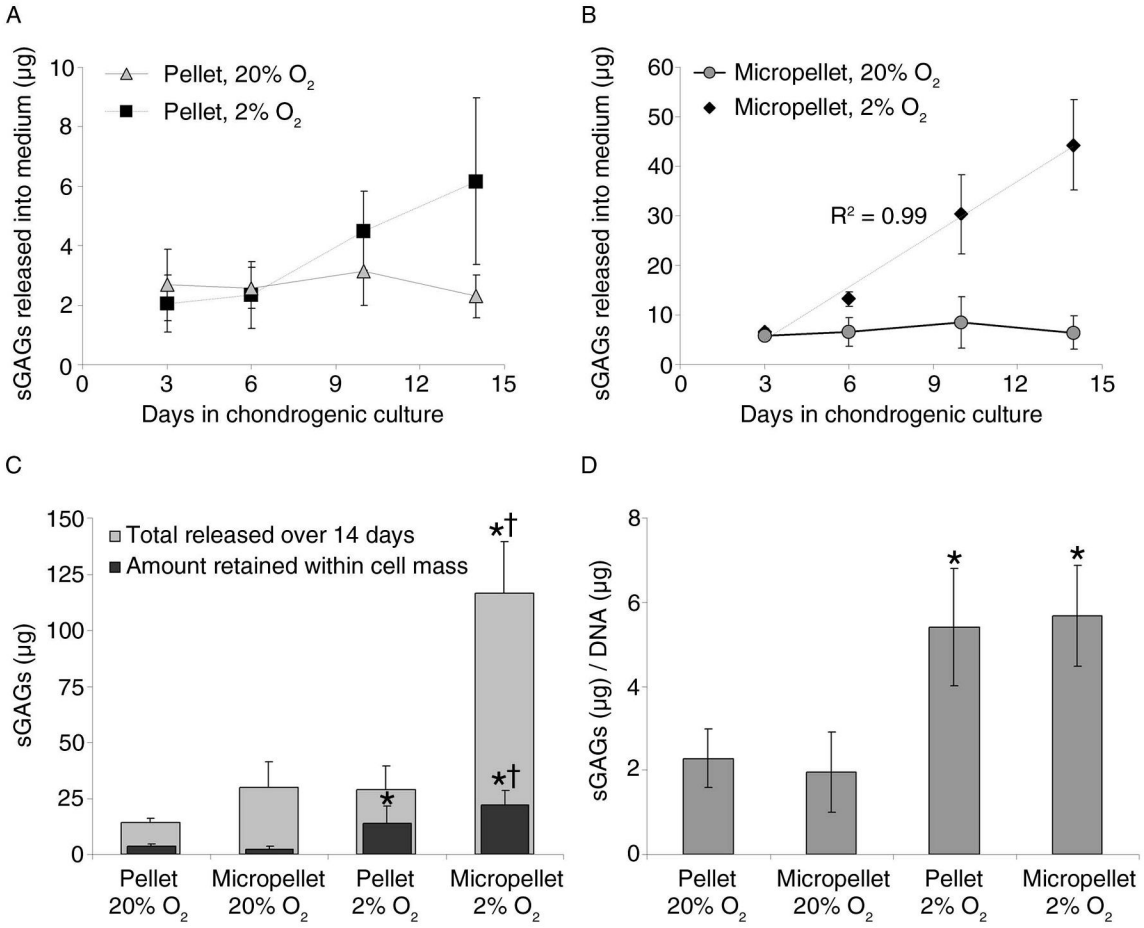

Figure 4.3. Production of sGAGs by human BM-MSC pellets and micropellets differentiated under 20% and 2% O_2. The release profile of sGAGs over 14 days was determined by the quantity released into the supernatant between medium exchanges for (**A**) conventional pellet cultures and (**B**) micropellet cultures. (**C**) The total amount of sGAGs produced over 14 days and the amount retained within the pellets and micropellets after 14 days. (**D**) The amount of sGAGs that was retained within the pellets and micropellets normalized to DNA. All values are mean ± standard deviation for n=10 samples from 3 independent experiments. Statistical significance was determined by ANOVA with Tukey post hoc tests. * $p < 0.05$ compared to 20% O_2 pellets; † $p < 0.05$ compared to 2% O_2 pellets.

4.5.3 Matrix Distribution

After 14 days the distributions of proteoglycans and collagens in the pellets and 2% O_2 micropellets were visualized using toluidine blue and polarized light imaging of picrosirius red, respectively. Due to the small size of remaining 20% O_2 micropellets, we did not cryosection samples for this evaluation. In pellets, staining of proteoglycans was heterogeneous with the darkest staining around the periphery while 2% O_2 micropellets exhibited homogeneous staining (Fig. 4.4A). Throughout the 2% O_2 micropellets the cells appeared as round, chondrocyte-like cells embedded within lacunae. The distribution of collagen fibers was similar in the 20% O_2 pellets and 2% O_2 pellets, with thicker fibers aligned along the periphery and random thin fibers in the central region (Fig. 4.4B, 4.4C). A random meshwork of fibers with no regional organization was observed surrounding the cells in the 2% O_2 micropellets. The collagen matrix surrounding the differentiating cells was also assessed by visualizing collagen I and collagen II using IF. Collagen I was detected throughout pellets differentiated under both oxygen environments and consistent with toluidine blue staining, collagen II was only detected in areas along the periphery (Fig. 4.5A, 4.5B, 4.5C). In contrast, positive immunohistochemical stainings of both collagen I and collagen II were visualized essentially throughout the matrix of 2% O_2 micropellets, although collagen I was generally more intense in the inner regions.

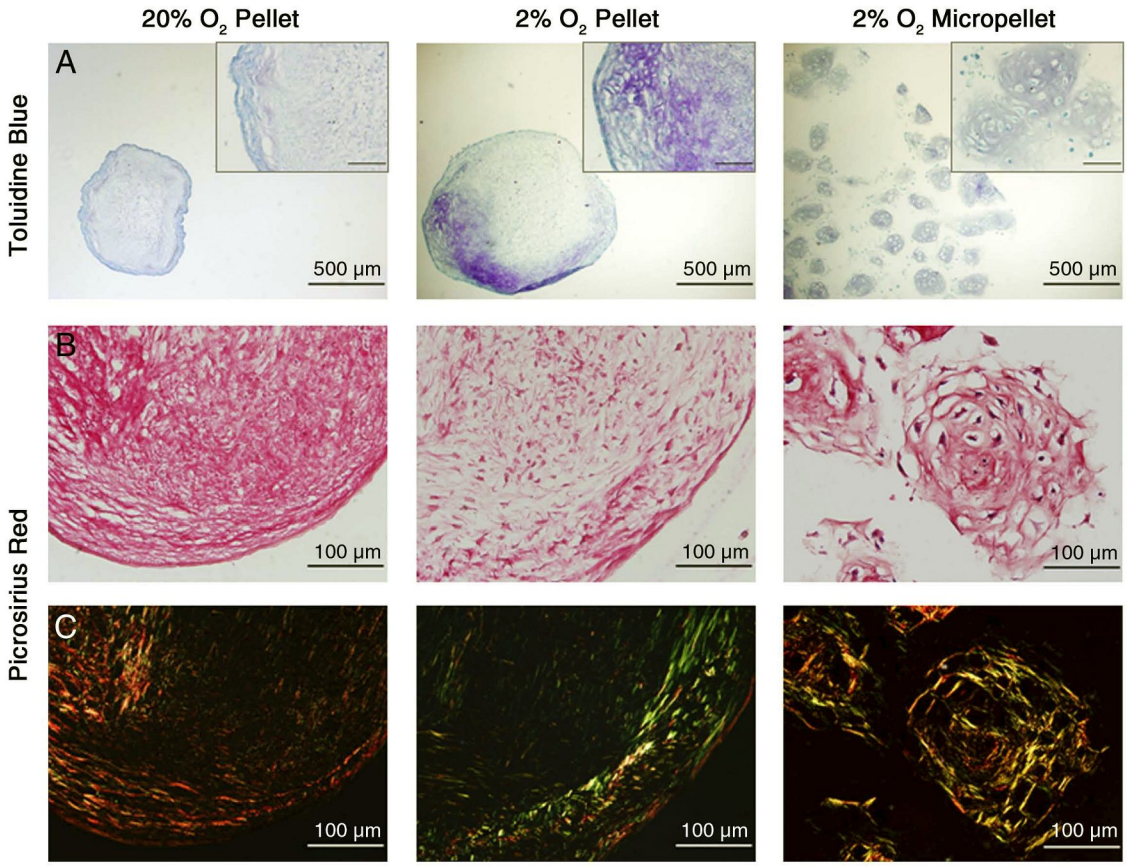

Figure 4.4. Distribution of proteoglycans and organization of collagen fibers in 20% O_2 pellet, 2% O_2 pellet and 2% O_2 micropellet cultures of human BM-MSCs in chondrogenic medium for 14 days. Fixed cryosections were stained with (**A**) toluidine blue for proteoglycans or (**B**) picrosirius red for collagen and viewed under normal bright field. (**C**) Birefringent collagen fibers of the picrosirius red-stained sections were visualized with polarized light microscopy. For inserted images, scale bar = 100 µm.

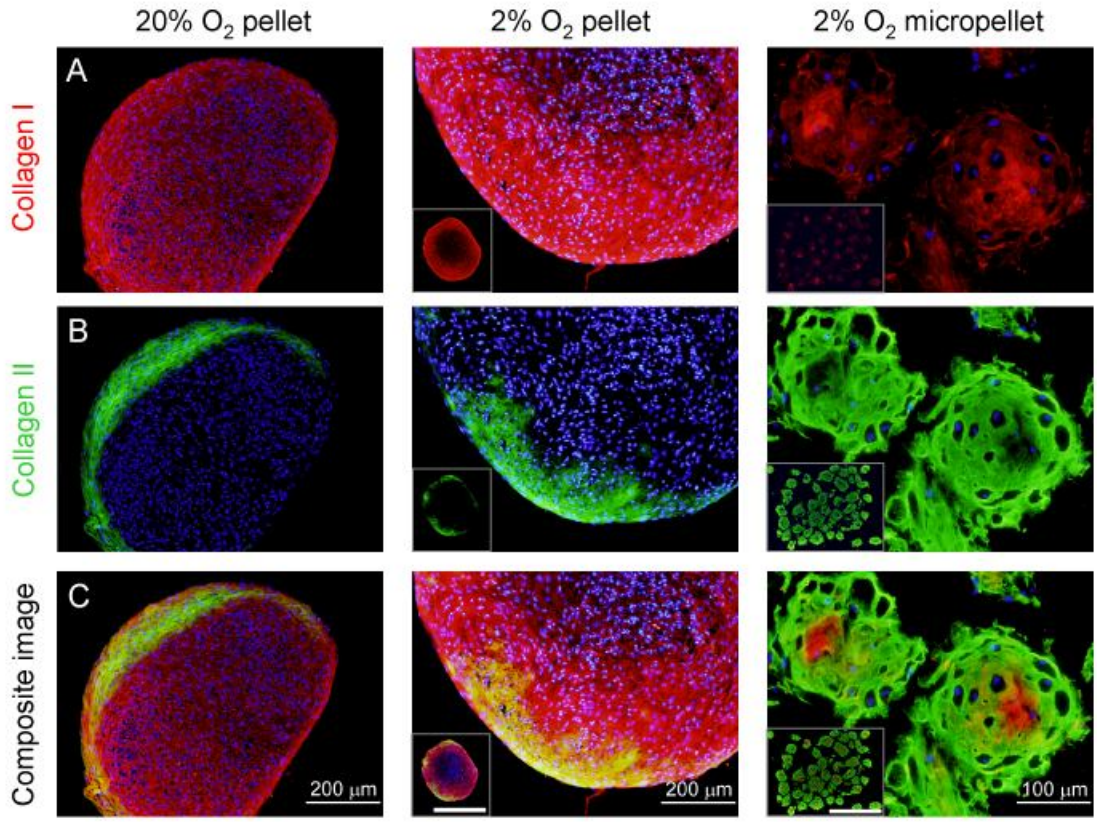

Figure 4.5. Localization of collagen in 20% O_2 pellet, 2% O_2 pellet and 2% O_2 micropellet cultures of human BM-MSCs maintained in chondrogenic medium for 14 days. Fixed cryosections were double-stained with (**A**) anti-collagen I and (**B**) anti-collagen II antibodies and with Hoechst 33342 as a counterstain for nuclei. Positive immunolocalization of collagen appears as red (collagen I) or green (collagen II) in the extracellular matrix, and nuclei are blue. Negative controls where the primary antibodies were omitted were used for background correction. (**C**) Composite images of (A) and (B) that were digitally processed using ImageJ software. The overlapping regions (yellow) indicate co-localization of collagens I and II. For inserted images, scale bar = 1 mm.

4.5.4 Gene Expression

The extent of chondrogenesis was distinguished by quantifying the relative gene expression of collagen II, aggrecan, and SOX9. We also evaluated a variety of genes more prevalent in MSCs, fibrocartilage, hypertrophic chondrocytes, and osteoblasts: collagen I, versican, collagen X, Runx2/Cbfa1, and osteocalcin.

After 14 days of chondrogenic induction, qPCR analysis revealed no significant difference in SOX9 expression between 2% O_2 and 20% O_2 pellets (Fig. 4.6A). SOX9 in

2% O_2 micropellets, meanwhile, showed an average increase of 7.5-fold over 20% O_2 pellets and 3.0-fold over 2% O_2 pellets. Aggrecan was increased 12-fold and 350-fold in 2% O_2 pellets and 2% O_2 micropellets, respectively, compared to 20% O_2 pellets (Fig. 4.6B). Collagen II was increased on average 1,500-fold in 2% O_2 pellets and 33,000-fold in 2% O_2 micropellets compared to 20% O_2 pellets (Fig. 4.6C). Aggrecan and collagen II were increased on average 22-fold and 785,000-fold, respectively, in 2% O_2 micropellets compared to monolayer MSCs (Fig. 4.7). We screened some of the 20% O_2 micropellet samples for aggrecan and collagen II gene expression and found these to be expressed at similar levels as in 20% O_2 pellets and taking into consideration the sGAG results, did not continue with the full panel of genes for 20% O_2 micropellets.

Collagen I was not significantly different between pellet cultures but increased 4.2-fold in 2% O_2 micropellets compared to 20% O_2 pellets (Fig. 4.6C). Comparing 2% O_2 micropellets to monolayer MSCs using GAPDH revealed a 2.2-fold increase (Fig. 4.7), meaning that the monolayer MSCs expressed collagen I at an intermediate level between pellets and 2% O_2 micropellets. Versican, on the other hand, was not significantly different among the differentiated conditions (Fig. 4.6B). Collagen X followed the trend of collagen II although it was highly variable among samples even within the same donor. Still, there was a significant 21-fold increase in collagen X in 2% O_2 pellets and 1,600-fold in 2% O_2 micropellets compared to 20% O_2 pellets (Fig. 4.6C). Despite this trend, there was no significant difference in Runx2/Cbfa1 or osteocalcin gene expression in 2% O_2 micropellets compared to 20% O_2 pellets (Fig. 4.6A, 4.6D), although Runx2/Cbfa1 was significantly lower in 2% O_2 pellets compared to micropellets.

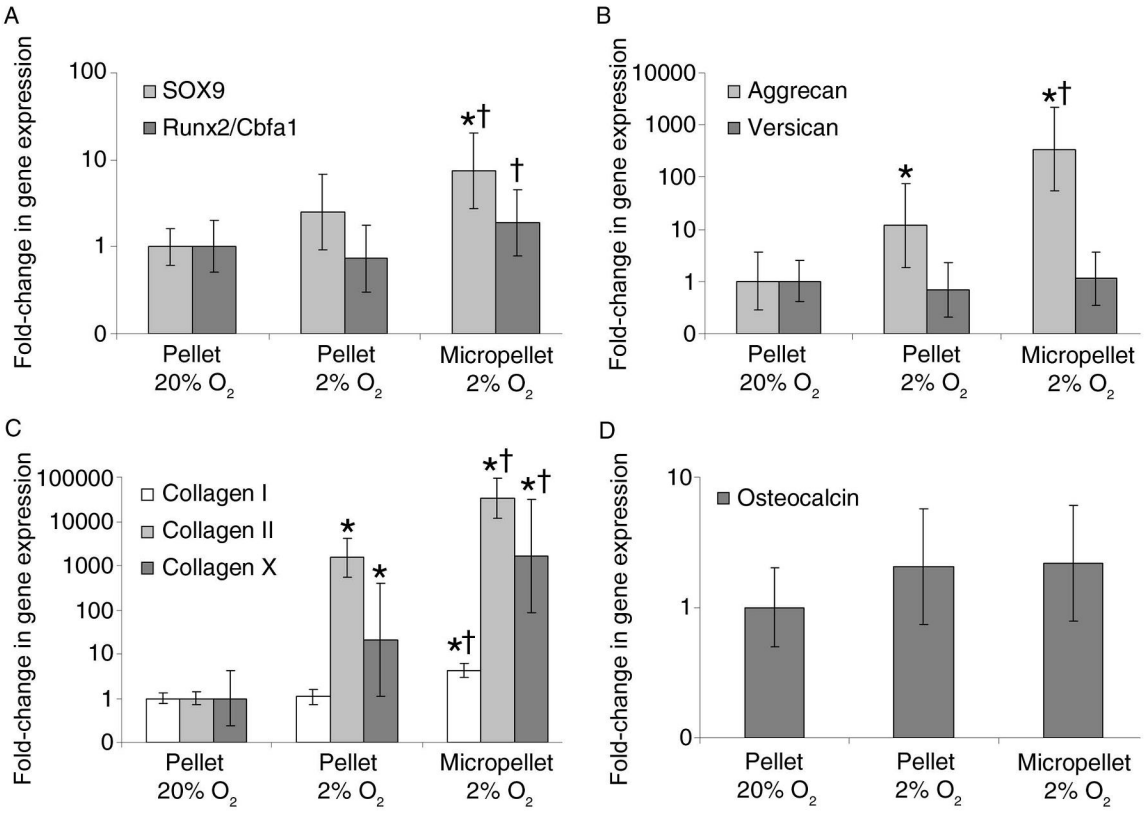

Figure 4.6. Fold-change in mRNA levels of common markers for chondrogenesis, hypertrophy, and osteogenesis in human BM-MSC pellets and micropellets differentiated under 2% O_2 compared to conventional 20% O_2 pellets. After 14 days in chondrogenic culture the relative gene expression levels of (**A**) the transcription factors SOX9 and Runx2/Cbfa1, (**B**) the proteoglycans aggrecan and versican, (**C**) collagens I, II, and X, and (**D**) the bone-forming marker osteocalcin were analyzed by qPCR. All values are the mean fold-change relative to 20% O_2 pellets normalized to cyclophilin A for n=7 samples from 3 independent experiments. Error bars represent 95% confidence intervals. Statistical significance was determined by ANOVA with Tukey post hoc tests. * $p < 0.05$ compared to 20% O_2 pellets; † $p < 0.05$ compared to 2% O_2 pellets.

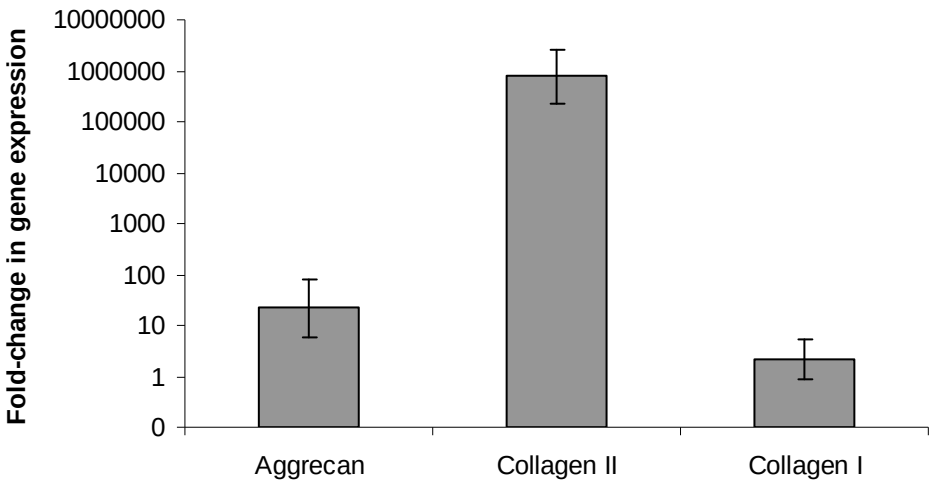

Figure 4.7. Fold-change in mRNA levels of collagen I, aggrecan, and collagen II in human BM-MSC micropellets differentiated under 2% O_2 compared to undifferentiated monolayer human BM-MSCs expanded under 2% O_2 environment. All values are the mean fold-change relative to 2% O_2 monolayers at day 0 of chondrogenic induction normalized to GAPDH for n=7 differentiated samples from 3 independent experiments. Error bars represent 95% confidence intervals.

4.6 Discussion

In this study, we investigated chondrogenesis of human BM-MSCs in conventional pellet culture compared to micropellets and the effects of oxygen tension in these different aggregates. Forced aggregation of MSCs is often used for chondrogenic induction in order to recapitulate critical signals of developmental chondrogenesis that arise during condensation, such as N-cadherin engagement. The importance of aggregation has recently been further explained by the role of Jagged-1-mediated Notch signaling in the chondrogenesis pathway [392]. Notch signaling, which is activated by cell-cell contact, must be initiated for human BM-MSCs to begin chondrogenesis but must later be switched off for differentiation to continue [324]. TGF-β3 was recently discovered to downregulate Notch gene expression and protein levels in human BM-MSCs, resulting in the SOX9-mediated upregulation of collagen II [393]. Thus, TGF-β family members may be key regulators resulting in the transience of Notch signaling that is critical for chondrogenesis of human BM-MSCs. Given that hypoxia reportedly enhances Notch signaling in other cell types [394], oxygen tension may also be a crucial factor in this pathway. We hypothesize that the benefits of low oxygen on *in vitro* MSC

chondrogenesis may be abrogated somewhat by the limitations of current chondrogenic protocols. Specifically, we believe that the mass transport properties and self-induced oxygen gradient of macroscopic pellet culture introduce uncontrollable variables that are obstacles to robust chondrogenesis necessary for tissue engineering applications.

The most critical known soluble factors for chondrogenic differentiation of MSCs range in molecular weight from 0.39 kDa (dexamethasone) to 25 kDa (TGF-β). Mass transport studies of tissue-engineered cartilage constructs have shown a relationship between increasing tissue density and decreasing diffusivity, even for molecules as small as glucose (0.18 kDa) [395-397]. Some studies suggest that cell growth in certain constructs is not limited by mass transport [398, 399], however, diffusivities in commonly used scaffolds have been found to be notably higher than those in native articular cartilage [397]. In pellet culture, the microarchitecture has been observed to resemble that of articular cartilage, with collagen fibers aligned parallel to the surface at the surface and perpendicular to the surface in the deeper layers [317, 322, 400]. Interestingly, in articular cartilage, Leddy and Guilak discovered that small and large molecular weight dextrans (3 and 500 kDa) had their greatest diffusivities through the surface zone, while mid-range dextrans (40 and 70 kDa) had lower diffusivities through the surface zone than through the middle and deep zones [401]. Over the course of differentiation of pellet-cultured MSCs, it is likely that matrix accumulation results in the diffusivity approaching that of articular cartilage. Therefore, strategies which minimize the mass transport limitations in MSC chondrogenesis have the potential to maximize the efficiency and homogeneity of differentiation. The wells of AggreWell plates each consist of approximately 1,200 microwells and allow for the creation of very small diffusional distances in adjacent but physically separate microscopic aggregates. Using the assumption that cells form perfect spheres of an equal initial total volume as a single perfect sphere of cells, distributing the cells across the AggreWells would decrease the radius of aggregates by approximately 11 times. This would result in an approximate 11-fold increase in the surface area-to-volume ratio which would substantially enhance mass transport.

Over 14 days of chondrogenic induction, BM-MSC micropellets cultured in AggreWell plates under a 2% O_2 environment demonstrated substantially increased chondrogenesis over conventional pellet cultures differentiated under both 2% and 20% O_2. While the endpoint for analysis was at 14 days we monitored development over the

course of chondrogenic induction morphologically and by quantifying the release of sGAGs. The accumulation of sGAGs begins in the early stages of MSC chondrogenesis [314] and the quantification of these proteoglycan modifications can give an indication of aggrecan production. Aggrecan is largely responsible for providing the compressive stiffness of articular cartilage [159] and obtaining cartilaginous tissues *in vitro* with similar aggrecan composition remains a challenge [317, 402]. In accordance with results seen in pellets of normal ACs [392], our BM-MSC pellets under 2% O_2 retained more of their total sGAGs within the cell mass and correspondingly produced more per DNA than the 20% O_2 pellets. These 2% O_2 pellets were also noticeably larger when viewed with the naked eye, a result others have reported for low oxygen-preconditioned and low oxygen-exposed pellet cultures of MSCs and ACs, which is likely primarily a product of increased proteoglycan content [376, 377, 392, 403]. The release profiles for 20% O_2 micropellets were generally elevated over pellet cultures but per DNA within the cell mass, they performed no better than conventional 20% O_2 pellets. The difference in sGAG production over 14 days in 2% O_2 micropellets, on the other hand, was striking. These micropellets swelled in size like their macroscopic counterparts under 2% O_2 and had a highly linear increase in sGAG release over the entire 14 days. Although 2% O_2 micropellets retained a lower fraction of their total than 2% O_2 pellets, the total sGAG production was so significantly elevated that the quantity of sGAGs per DNA within the mass was similar.

We histologically assessed the distributions of proteoglycans as well as collagen in the 20% O_2 pellets, 2% O_2 pellets, and 2% O_2 micropellets. In conventional pellets collagen II and proteoglycans are typically observed either in the center [202, 203, 314, 324] or at the periphery [313, 314, 316, 317, 322, 323]. Although, due to multiple variables between these studies, it is difficult to discern the reasoning for the different patterns observed, the fluctuating mass transport properties of the differentiating pellet may play a role. In larger pellets, central necrosis has even been observed, possibly due to extreme diffusive transport limitations [404]. In this study, both 20% and 2% O_2 pellets displayed mostly peripheral staining of proteoglycans and collagen II but showed collagen I throughout. In agreement with the biochemical analyses, toluidine blue showed the faintest staining for proteoglycans within the pellet mass for 20% O_2 pellets. Pellets differentiated under a 2% O_2 environment showed more peripheral staining of proteoglycans and clear regions of heavy proteoglycan accumulation. Although

micropellets were not a perfectly uniform size the distribution of proteoglycans and collagens appeared homogeneous. The micropellets also displayed random orientation of collagen fibers similar to the middle to deep zones of normal articular cartilage [159, 317, 405]. There were no structurally aligned fibers around the periphery of the micropellets as was seen in conventional pellets and as has been observed by others [317]. Furthermore, while collagen I was variably distributed in the micropellets, collagen II appeared throughout micropellets after only 14 days with TGF-β1.

Analysis of cartilage-specific genes using qPCR revealed collagen II and aggrecan to be significantly upregulated in 2% O_2 pellets but further increased in 2% O_2 micropellets along with SOX9. Collagen II in 2% O_2 micropellets was elevated 33,000-fold over 20% O_2 pellets and approximately 785,000-fold more than undifferentiated BM-MSCs after 14 days. In 2% O_2 micropellets with 10 ng/ml TGF-β1, we measured increases in collagen II expression of similar magnitudes as have been reported for normoxic pellet cultures pre-stimulated with FGF-2 and supplemented with 100 ng/ml of the purportedly more potent TGF-β3 [324]. Due to the extremely high level of sGAGs produced in 2% O_2 micropellets compared to pellet cultures, in addition to aggrecan we also evaluated versican expression, which is high in dedifferentiated ACs but low in normal articular cartilage [145, 385]. Versican is also expressed in undifferentiated BM-MSCs [321] and an increase in its gene expression has been described as an early event in pellet culture chondrogenesis [314]. In this study, unlike aggrecan expression which increased on average 350-fold in 2% O_2 micropellets compared to 20% O_2 pellets, there was no significant difference in versican expression among the different conditions.

The inability to maintain high levels of collagen II expression relative to collagen I in primary ACs expanded *in vitro* potentially reduces their efficacy in ACI procedures [145, 188]. While collagen I is highly expressed in BM-MSCs [321] and extremely low in normal ACs [145, 146], its average expression during pellet culture chondrogenic induction of BM-MSCs has been observed by some to increase along with collagen II and collagen X over the long term [236, 315, 316]. We found that the average collagen I expression was significantly higher in 2% O_2 micropellets compared to 20% and 2% O_2 pellet cultures, both of which had decreased levels compared to monolayer BM-MSCs. However, we think that with continued induction beyond 14 days the pellets would have continued to increase their average collagen II and collagen X expression as well as their average collagen I expression.

Perhaps the principal challenge of using BM-MSCs for cartilage repair will be the ability to obtain a stable chondrogenic phenotype. In this study, we evaluated the hypertrophic and osteogenic markers collagen X, Runx2/Cbfa1, and osteocalcin. Changes in collagen X expression among conditions mirrored the changes seen with collagen II, as has been reported previously with conventional pellets [236, 314, 317-319, 376]. This could be a significant obstacle with BM-MSCs as Pelttari et al showed that ectopic implantation of *in vitro* differentiated BM-MSC pellets in SCID mice led to calcification and vascular invasion at the implant site, whereas no such problems were observed with chondrocyte-derived pellets [236]. However, we believe that our micropellet platform will allow for better control when investigating methods to reduce collagen X expression and likewise, collagen I expression.

Collagen X expression in hypertrophic chondrocytes is regulated by Runx2/Cbfa1 [281, 286, 288], a critical transcription factor for osteoblast differentiation and bone formation [300, 406, 407] and an important regulator of human BM-MSC osteogenic differentiation [408, 409]. Despite greatly increased levels of collagen X, there was no significant difference in Runx2/Cbfa1 gene expression compared to 20% O_2 pellets. However, while increased Runx2/Cbfa1 activity is critical for osteogenesis of human BM-MSCs, its mRNA expression may not change even during osteogenic differentiaton [408]. We therefore examined expression of another Runx2/Cbfa1 target, osteocalcin, as it is regulated during osteogenesis and has been indicated as a hypertrophic marker in chondrocytes and in human BM-MSC pellet cultures [316, 410-413]. Despite the apparent advanced stage of the 2% O_2 micropellets, there was no significant difference in osteocalcin expression among conditions. While 14 days may be too early to see such changes even in the micropellets, in addition to observing reduced osteogenesis of human MSCs under prolonged low oxygen tension [372], others have noted that even temporary hypoxic exposure inhibits osteogenic differentiation [378]. Consequently, we believe that with further optimization micropellets under low oxygen tension may offer a means to reach a stable chondrogenic phenotype.

4.7 Limitations

In this study, we evaluated the micropellet platform using microwell surfaces manufactured and sold commercially. Thus, while we could vary the theoretical number of cells per microwell, we were limited to the specified dimensions of microwells by the

premade inserts. Although each well of the AggreWell plates consists of approximately 1,200 microwells, each 400 μm in diameter, micropellets across microwells were not a perfectly uniform size likely due to edge effects and the method of microwell insert adhesion to the plates and possibly exasperated by the long-term culturing with multiple medium changes. The variation in micropellet size was evident upon initial deposition and appeared, at least initially, to be due to the uneven application of the adhesive agent used to affix the microwell inserts to the well bottoms. Microwells having few cells initially deposited were most prominent near the edges of the wells and these microwells were often seen as having adherent, spread cells even at 14 days. Additionally, the AggreWell plates are advertised for their use with embryonic stem cells and are designed for short-term use, specifically for the initial formation of homogeneous embryoid bodies before transferring to low adherence culture dishes. While medium was removed by gentle aspiration and loss of micropellets during feeding did not appear significant, by 14 days most micropellets were only loosely adherent to the underlying surface if at all. Also, at these late time points it sometimes appeared that micropellets from adjacent microwells would become fused to each other. Therefore, the shallow depth of the microwells could make medium changes without loss of micropellets and maintenance of individual micropellets particularly difficult for lengthier studies. As the gene expression data was generated from RNA collected from the entire well, this represents the average expression from aggregates of various sizes, including those that did not aggregate at all. However, we expect that improved deposition resulting in a higher fraction of the cells being a part of micropellet aggregates would only further enhance the chondrogenic changes observed.

For this study establishing the micropellet platform, we considered chondrogenic characterization at only one late time point. From our results, we speculate that the differentiation is not only more homogeneous but also more rapid. However, a detailed time-based analysis including early timepoints is necessary to confirm this and to elucidate the extent of such differences. Additionally, we quantified only the changes in mRNA levels and not corresponding protein expression. Western blots have been utilized to evaluate protein expression in pellet cultures and this technique could be readily applied to micropellets. This will be important to further evaluate the chondrogenic response of BM-MSCs in the micropellet platform. So while our IF images indicate a better distribution and correspondingly higher overall expression of collagen II

protein, quantitative methods for expression of relevant proteins will be needed to more comprehensively characterize the micropellet platform for MSC chondrogenesis.

4.8 Conclusion

Here we showed the benefit of low oxygen tension during chondrogenic induction of human BM-MSC using both conventional pellet culture and a new micropellet culture. The benefits of low oxygen tension appear to be maximized by creating very small aggregates which limit mass transport and oxygen diffusion gradients. While the undesired collagen X was greatly upregulated in 2% O_2 micropellets, this problem persists in conventional BM-MSC pellet culture as well. Soluble factors such as chondroitin sulfate [414] and PTHrP [206, 323] will likely be the most commonly investigated solutions to this problem and as shown here, a micropellet system may maximize the efficiency of such strategies. With a view towards future tissue engineering, we believe the benefits of performing MSC chondrogenesis in this way is to date unparalleled amongst scaffold-free methods. Homogeneous differentiation of the cell population can be achieved more rapidly and the physical nature of the chondrogenic micropellets should make them easy to integrate with a variety of stem cell-based cartilage repair technologies such as intra-articular injections, hydrogels, and scaffolds.

CHAPTER V

Summary

Tissue engineering solutions are of particular interest for injured tissues with deficient endogenous cellular repair such as atherosclerotic lesions of the vascular wall and chondral lesions of the synovial joints. These problems, when they do require surgery, are commonly treated with autologous tissue transplants that may cause donor-site problems or, alternatively, with prosthetics that often have inferior long-term outcomes. Tissue engineering techniques for these injuries have yet to yield solutions that properly reproduce the relevant specialized tissues capable of restoring function and providing long-term protection against recurring degeneration. Tissue engineering approaches to vascular injuries often utilize biomaterials that provide the structure and mechanical strength and seek to promote an endothelial monolayer covering through *in vitro* seeding or *in vivo* induction of migration onto the biomaterial. The predominant physical challenge to vascular engineering is shear stress from the flowing blood. Cartilage engineering, on the other hand, may or may not utilize biomaterial scaffolds as it seeks to reconstruct a three-dimensional mass of chondrocytes and specialized matrix. Here the primary physical force that will be exerted on the implanted tissue is compression. Thus, treatment of atherosclerotic and chondral lesions both stand to benefit greatly from advances in tissue engineering but the nature of these lesions present distinctly different challenges for regenerative strategies.

The two parts of this dissertation focused on the limitations of current cell-based therapies for vascular and cartilage engineering, specifically with respect to relevant stem and progenitor cell populations. Both vascular engineering with EPCs and cartilage engineering with MSCs are limited by the efficiency and rate of differentiation of the primitive cells to functional cells resembling ECs and ACs. Two separate approaches were devised as methods compatible with current tissue engineering interventions. The

methods described here were conceived of with consideration to the compartmentalization of the relevant stem/progenitor cells as well as relevant environmental factors. EPCs circulate in peripheral blood and upon seeding to surfaces of vascular grafts would be exposed to shear stress. Abrupt exposure to shear stress is a classic problem for vascular engineering with statically seeded ECs prone to being washed away. The proposed strategy for vascular engineering with EPCs is therefore to take advantage of their location in circulation and to capture them directly from flow to solid surfaces, thus in effect immediately conditioning them to shear stress. MSCs that are capable of chondrogenesis are physically separated from cartilage, residing in the low oxygen environment of the bone marrow. The aggregation of MSCs is thought to be important for chondrogenesis but the formation of large aggregates may limit diffusion of critical growth factors resulting in heterogeneous differentiaton. As cartilage is also a hypoxic environment, the proposed strategy for cartilage engineering is to transfer MSCs from bone marrow to cartilage defects, keeping them under low oxygen during the process and differentiating the cells through microscopic aggregates hypothesized to allow homogeneous control of differentiation. The studies described in this dissertation were designed to answer critical questions of feasibility with regard to these strategies and to lay the foundation for further development.

Vascular prosthetics engineered to stimulate endothelialization via capture of EPCs is an idea that has been implemented by others using anti-CD34 mAb. Information regarding the dynamics and specificity of capture is lacking and the choice of CD34 as a target molecule may not be the most valuable of the posited EPC markers. KDR is unique among EPC markers, being involved in mediating ligand-dependent and ligand-independent atheroprotective functions of ECs and presumably regulating EPC differentiation. To evaluate the suitability of using anti-KDR mAb to capture flowing KDR^+ cells, HUVECs were used as they widely express KDR. Anti-KDR mAb was found capable of capturing HUVECs at low shear rates and to do so specifically by the antigen-binding domains. Because KDR^+ cells are a small fraction of PBMNCs, we evaluated the effects of a large proportion of cells lacking surface KDR (BaSMCs) on the capture of target cells. The efficiency of anti-KDR antibody-mediated HUVEC capture was determined to be unaffected whether flowing HUVECs were in the presence or absence of BaSMCs. We also found that capture could be increased substantially by using protein G to orient the antibody. These results provide hope that anti-KDR mAb may be

used for capture surfaces as anti-CD34 mAb has previously been utilized. Further studies are required to optimize the surface for use with blood cells, for effectiveness at higher shear rates, and for promotion of functional properties of captured cells.

The ability to use MSCs in cartilage repair strategies such as ACI will be improved with the development of efficient and controlled chondrogenesis methods. A proven method for driving *in vitro* chondrogenesis is to form MSC aggregates in the presence of defined medium supplemented with chondro-inductive factors such as TGF-β. Recent developments in embryoid body formation have provided a means for miniaturizing aggregates and the effectiveness of such a technology for enhancing chondrogenesis of human BM-MSCs was evaluated in this study. Due to the promising indications but lack of clarity regarding the role of oxygen tension during chondrogenesis of human BM-MSCs, the oxygen environment was varied between normoxic (20% O_2) and hypoxic (2% O_2) for this new culture system as well as for conventional pellet culture. It was shown that a long-term low oxygen environment during chondrogenic induction has beneficial effects on the differentiation of human BM-MSCs in conventional macroscopic pellets. Furthermore, creating smaller cell aggregates under low oxygen tension resulted in substantial increases in the changes characteristic of chondrogenic differentiation compared to conventional pellet cultures in both environments. Specifically, it was shown that chondrogenic induction of BM-MSC micropellets formed under low oxygen tension results in considerably increased sGAG production, uniform distribution of matrix components, and enhanced expression of genes associated with BM-MSC chondrogenesis. Thus, we have developed a new method for enhanced chondrogenic differentiation of BM-MSCs that possesses properties ideal for incorporation with current platforms for cartilage repair. Further studies are required to more completely characterize the chondrogenic response, to optimize parameters promoting an expression profile characteristic of permanent cartilage, and to develop integration strategies for harnessing the potential of micropellets for tissue engineering applications.

Engineering functional endothelial monolayers and three-dimensional neocartilage presents distinct challenges with regard to geometry, the compartmentalization of the relevant precursor cells, and environmental factors. It is hoped that these studies answer critical questions regarding feasibility of the proposed

EPC and MSC-based tissue engineering strategies and lay the foundation for further development towards clinical relevancy.

CHAPTER VI

Future Work

The studies detailed here provide the groundwork for the use of future generations of the platforms in tissue engineering applications for the relevant injuries. The results obtained provide a reason for optimism with respect to the platforms' utilities but also raise many questions, opening the doors to numerous potential projects designed to further characterize outcomes and to elevate the methodology. A few of the more immediate questions and ideas for future directions are presented here.

6.1 Anti-KDR Antibody Endothelial Capture Surfaces

6.1.1 Modifications for Use with Blood Cells

In order for an antibody-mediated capture surface to be utilized for blood-contacting devices, the potential for nonspecific adherence must be minimized. In fact, the development of more sophisticated surfaces becomes necessary already when switching from HUVECs and BaSMCs to purified PBMNCs in our *in vitro* system. For example, in the flow regime studied here we observed that PBMNCs readily adhered to BSA-coated surfaces but that the surfactant Pluronic® F108 effectively eliminated PBMNC adhesion to surfaces even in static conditions. While coating with BSA may make a suitably nonadhesive surface for many cell types, leukocytes adhere to BSA, and even more so to denatured BSA, through their β_2-integrins [415]. Pluronic F108 can passivate hydrophobic surfaces, such as polystyrene, in a matter of minutes and in preliminary flow experiments we observed that it makes a suitable alternative blocking agent to BSA for both HUVECs and PBMNCs in the flow chamber utilized here. Adhesion of HUVECs perfused over Pluronic F108-coated polystyrene was negligent and adhesion to anti-KDR mAb-coated surfaces was not negatively affected by this

blocking agent. However, while flowing PBMNCs did not adhere to Pluronic F108-coated surfaces, they were observed to adhere to adsorbed antibodies of both anti-KDR and anti-TGF-β with similar frequency, indicating nonspecific capture. Additionally, surfaces coated with protein G without antibodies resulted in substantial arrest of PBMNCs, even with this blocking agent. Whether applying anti-KDR mAb to protein G could prevent nonspecific adherence is unknown; it is possible that orienting the antibody and saturating protein G's immunoglobulin-binding sites could sterically prevent adhesion of cells to the underlying protein G. This seems unlikely as protein G is nonspecifically adsorbed in our system but determining this will require less direct methods than were used for the model system. In the model system, two populations of cells were available, one which expressed KDR on the surface and one which did not. These different populations were stained with different dyes independently and then mixed together for flow studies. For a group of cells that are a heterogeneous mixture from the beginning, such a method is not possible. Using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) to separate KDR^+ cells could substantially decrease the ability of these cells to then adhere to anti-KDR mAb-coated surfaces. One way to evaluate the specificity of capture from PBMNCs would be to use FACS (or MACS) to deplete PBMNCs of KDR^+ cells by collecting the KDR^- fraction and subjecting this population to flow over anti-KDR mAb-coated surfaces. The specificity could then be evaluated by subjecting mock-sorted PBMNCs (i.e. FACS with antibody that does not bind surface proteins of PBMNCs) to flow over these surfaces.

Another immobilization strategy, such as streptavidin-mediated orientation of biotinylated antibody, could also be considered as an alternative to protein G-mediated immobilization. Due to the symmetry of the streptavidin tetramer's biotin-binding subunits, immobilization of biotinylated molecules can be accomplished by modifying surfaces with biotin, adding streptavidin, and then the biotinylated molecule. Already others have begun investigating techniques to modify PTFE with biotin so that streptavidin can be linked to the surface, suggesting such a method could be used for immobilization of heparin [416]. The biotin-streptavidin linkage is one of the strongest noncovalent biological bonds known ($K_d = 10^{-15}$ M^{-1}) [109] and biotinylated antibodies can thus be strongly bound to streptavidin-coated surfaces. Peluso et al described how the conserved N-link glycosylation sites of IgG Fc chains can be specifically biotinylated and, alternatively, how Fab fragments can be separated and biotinylated at the reduced

cysteine thiol on the cleaved end [348]. When immobilized via biotinylated Fc chains with the Fab fragments directed away from the surface, the analyte-binding capacity was increased over surfaces with randomly immobilized antibodies and the effect was even more dramatic when utilizing cleaved, biotinylated Fab fragments. Jarvis and Bryers describe a process to synthesize such surfaces on glass [417] in a way which would be compatible with our *in vitro* flow chamber system. In this method, glass surfaces were silanized with 2% 3-aminopropyltriethoxysilane in toluene and then amine-reactive biotin was covalently linked to the silanized surface. The silanization process makes the glass hydrophobic so Pluronic F108 can be used as a blocking agent and the covalent linkage of biotin provides a specific and strong means for streptavidin to be immobilized. Displacement of adsorbed proteins by serum proteins is a potential problem with any blood-contacting surface (see Section 2.2.5). Using strategies which do not rely on nonspecific adsorption, such as biotin-streptavidin-mediated immobilization, therefore also have greater potential for translation to *in vivo* use.

Regardless of the strategy devised to immobilize antibodies, surfaces should also be evaluated for interactions with other cellular components of blood, such as neutrophils and platelets. Although antibody orientation would be expected to eliminate any possibility of Fc receptor-mediated adhesion, platelets have been reported to express functional KDR [418]. However, these receptors are thought to only become exposed with platelet activation and therefore it is not anticipated that oriented surface-bound anti-KDR mAb would stimulate platelet aggregation. Still, *in vitro* evaluation of platelet and neutrophil adhesion as well as the effect of other factors such as viscosity and packed red blood cells on specific cell capture would provide valuable insight for optimizing surfaces.

6.1.2 Development of Multi-functional Surfaces

After methods for antibody presentation are developed which are compatible with selectively capturing KDR^+ PBMNCs, further modifications will likely be needed to improve the utility of the surfaces for vascular devices. Here we have shown the arrest of cells by anti-KDR mAb at a shear rate of 50 s^{-1} but this is well below arterial levels of shear. To more effectively capture KDR^+ cells from PBMNCs at physiologically relevant shear rates, more complex surfaces may need to be developed. For example, mouse bone marrow-derived progenitors have been found to possess the functional membrane-

spanning proteins required for tethering and rolling and, as with leukocytes, this facilitates their adhesion from flow at sites of injury [338]. Modifying surfaces to present P-selectin amongst the capture antibodies is therefore a potential strategy to aid in capture at higher shear rates. As with surfaces presenting only anti-KDR mAb, there is at least potential for undesired platelet adhesion if surfaces are supplemented with P-selectin. Platelets express functional P-selectin glycoprotein ligand-1, although at levels estimated to be much lower than leukocytes [419]. Still, the potential for platelet interactions is something that should be considered whether this avenue or others is explored for increasing EPC capture.

While increasing the capture rate is ideal, the frequency of capture may be less important than providing an underlying substrate that promotes atheroprotective effects of captured cells. The captured cells will need to differentiate and, as noted by Rotmans et al in analyzing their subpar anti-CD34 mAb-coated grafts, the lack of a physiological subendothelial matrix may contribute to disappointing results [131]. Fibronectin and VEGF have been found to promote endothelial differentiation *in vitro* in a synergistic manner [420]. Utilizing these factors therefore is a natural inclination but their presentation to blood-contacting surfaces requires consideration of their effects on the other cellular components of blood. Platelets express integrins that bind fibronectin ($\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$) [421-423] but reportedly do not adhere to fibronectin at arteriole shear rates, binding optimally in the venous range at 300 s^{-1} [424]. Additionally, recent studies by McCarty et al suggest that soluble proteins in plasma prevent non-stimulated platelets from adhering to immobilized fibronectin [425] and previous studies indicate that fibronectin receptors are involved in platelet spreading on synthetic surfaces but probably not initial adhesion [426]. While β_2-integrins have been implicated as the primary integrins that mediate firm adhesion of leukocytes to inflamed ECs, leukocytes also express $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [337]. This would likely make the use of fibronectin in conjunction with roll-promoting molecules, such as P-selectin, impractical. The use of VEGF could also present challenges. As described in Section 2.2.3, monocyte and/or SMC chemoattractant activities of VEGF may have led to the poor long-term results observed in VEGF-coated grafts in animal models [55, 57]. Creative solutions, possibly at the expense of capture rate, will be required in order to provide sufficient cues to drive endothelial differentiation of captured cells without promoting accumulation of undesired cells.

6.1.3 Effects of Surface on EPC Atheroprotective Functions

Upon development of a surface that is capable of selectively capturing KDR^+ cells from PBMNCs, an essential question will be how that surface affects any atheroprotective properties these cells may possess. Patients receiving anti-CD34 mAb-coated stents receive double antiplatelet therapy for one to two months and this shortened duration compared to bare metal and drug-eluting stents is predicated on the notion that the anti-CD34-coated stent accelerates the formation of functional endothelium [130, 427]. However, to date no results have been published regarding the function of mature ECs on such surfaces, let alone EPCs. The recent case of late stent thrombosis reported for a patient who had received an anti-CD34 mAb-coated stent at least raises questions regarding the functional properties of the cellular layer formed [427]. For mature ECs it is well known that the production of two important atheroprotective agents, NO and PGI_2, is mediated by shear stress and the interactions of integrins with the underlying substrate are involved in this regulation (see Section 2.2.5). Therefore, promoting adhesion of cells by antibodies rather than normal physiological mechanisms may abrogate the synthesis of these products. Advanced design of surfaces for capture via anti-KDR mAb must take this into consideration. For future generations of capture surfaces, the shear-induced production of NO and PGI_2 by mature ECs on the surfaces should be evaluated as a basic part of characterization.

Assuming endothelial differentiation can occur on engineered capture surfaces, characterizing the function of mature ECs on these surfaces is an important consideration for assessing their long-term suitability. However, just as important is the question of the effects on early function of the captured cells. Vascular graft implants result in damage to tissue and an ensuing inflammatory reaction. Acute inflammation is characterized by the rapid accumulation of neutrophils followed by monocytes and its persistence leads to chronic inflammation [428]. Neutrophil infiltration has been implicated in neointimal thickening associated with arterial autografts and stenting [31, 32] and NO and PGI_2 are important inhibitors of neutrophil-endothelial interactions. The functionality of KDR^+ cells in this regard and specifically when adherent to antibody-coated surfaces, is therefore an important and interesting question. Perhaps due to the low frequency of presumed EPCs in circulating blood, to date there have been no published studies on the shear-induced production of NO and PGI_2 in freshly isolated or short-term differentiated EPCs. Studies on the production of these agents by EPCs have

utilized late outgrowth EPCs, those cells obtained from adhesion-selected PBMNCs following weeks of differentiation [84, 88, 90, 91, 93]. In a preliminary study, we attempted to quantify NO production by shear-exposed KDR^+ human PBMNCs following only 24 hours of adhesion to fibronectin in endothelial growth medium. KDR-sorted PBMNCs were either exposed to shear stress (20 dyn/cm^2) or left under static conditions and media samples were collected at 2 and 4 hours for quantification of NO with a commercial assay (Oxis International Inc, Foster City, CA). The results indicated that KDR^+ PBMNCs may respond to shear by increasing NO production (Fig. 6.1), however, confirmation of this will require further studies. In this study, PBMNCs were isolated and stained with FITC-conjugated anti-KDR mAb as described in Section 3.4.4, but were then transferred to a facility for sterile FACS. The KDR^+ cells retrieved by FACS for NO quantification studies were regularly a larger fraction of PBMNCs (11.7 ± 4.0% for N=4) than was determined by non-sorting flow cytometry analysis (3.4 ± 1.3% for N=5). It is possible then that Fig. 6.1 represents the shear-induced NO production by PBMNCs simply enriched for KDR^+ cells rather than a pure population of cells expressing this receptor. Future studies need to assess the flow-induced gene and protein expression of enzymes such as eNOS, inducible NOS, and COX-II in KDR^+ and KDR^- fractions of PBMNCs by more sensitive methods such as qPCR and Western blot analysis.

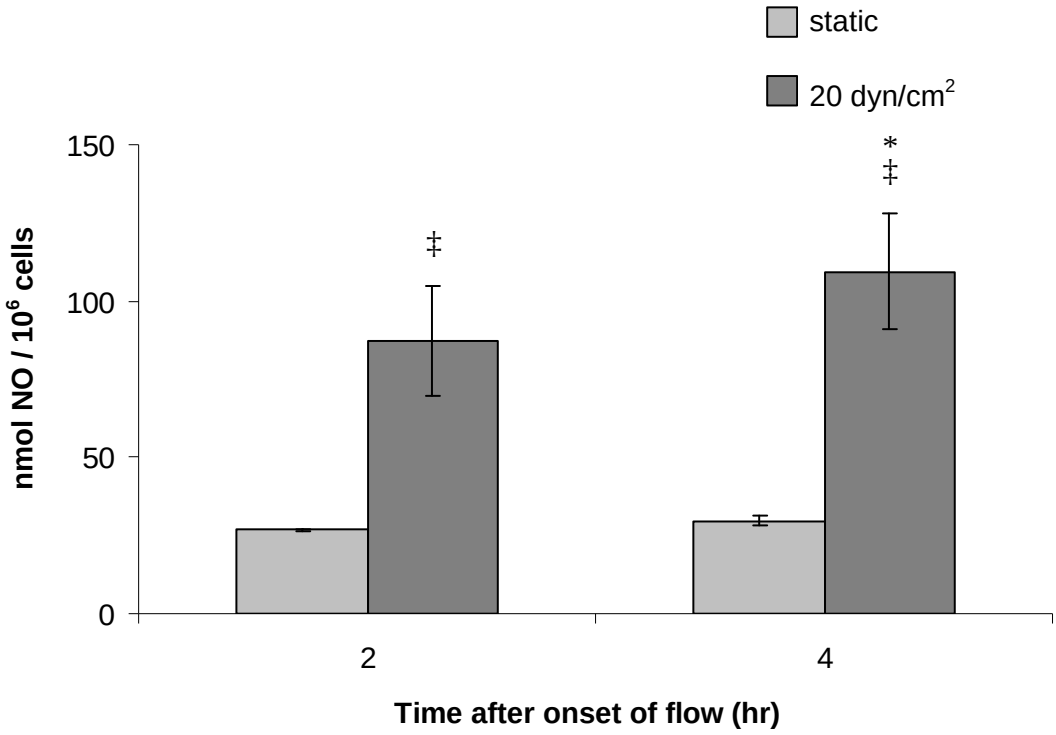

Figure 6.1. Fluid shear stress induces production of NO by adherent PBMNCs enriched for KDR^+ cells. PBMNCs were sorted for KDR^+ cells and the collected fraction plated on fibronectin-coated glass and allowed to adhere overnight. Cells were either exposed to shear stress (20 dyn/cm^2) using a peristaltic pump or left under static conditions and media samples were collected at 2 and 4 hours. NO production by shear exposed cells significantly increased from 2 hours (87 ± 18 nmol/10^6 cells) to 4 hours (109 ± 18 nmol/10^6 cells). NO production by shear-exposed cells was significantly greater than that of static cells measured simultaneously (27 ± 0 and 30 ± 2 nmol/10^6 cells at 2 and 4 hours, respectively). Values are mean ± SD for N=4 experiments. *P<0.01 compared to 2 hours (paired samples t-test); ‡P<0.01 compared to corresponding static measurement (independent samples t-test).

Due to the void of information regarding such atheroprotective responses in different populations of PBMNCs, it will be important to first assess this functionality on a surface known to be conducive to these responses in mature ECs (i.e. fibronectin). This will provide a baseline for NO and PGI_2 production to which antibody-coated surfaces can be compared and aid in optimizing future generations of surface design. The functional relevancy of these factors in graft patency is demonstrated by the discrepancy between saphenous vein and internal mammary artery grafts' early and long-term patency. Endothelium from internal mammary artery exhibit higher NO production and

PGI_2 synthesis and less neutrophil adhesion than saphenous vein ECs and this probably contributes to the superiority of internal mammary artery grafts [429, 430]. Because current evidence suggests that even differentiated EPCs release less NO and PGI_2 than mature ECs (see Section 2.2.4), it is of particular interest to design a surface that promotes synthesis of these agents if captured EPCs are to be considered an appropriate source for vascular graft lining.

6.2 Low Oxygen BM-MSC Chondrogenic Micropellets

6.2.1 Further Characterization of Current System

For the study establishing the micropellet platform, we investigated the hypothesis that this system would lead to a more homogenous distribution of cartilage-specific matrix and that the low oxygen environment would enhance this chondrogenic differentiation. To evaluate the degree of chondrogenic induction, the relative gene expressions of collagen II, aggrecan, and SOX9 were quantified. These are the classic genes used to characterize chondrogenesis of MSCs; however, they do not provide a complete picture of the quality of differentiation. It will also be interesting to evaluate the expression of other matrix genes associated with chondrogenesis such as COMP, collagen IX, and collagen XI. Cells make only 2-10% of the tissue volume of hyaline articular cartilage [158, 159] so the composition of the matrix of this specialized tissue is extremely important, as illustrated by ACI outcomes when fibrous and fibrocartilaginous tissue develops (see Section 2.3.3). The assembly of matrix components in a fashion which provides the unique mechanical properties of cartilage depends not only on an appropriate balance of the major components of aggrecan and collagen II, but also on the contributions of many other components. While collagen II is the major collagen of articular cartilage, COMP and the proteoglycan decorin have roles regulating macrofibril formation to which collagens IX and XI also contribute, lending to the tensile properties of the tissue; retention of aggrecan in the matrix, meanwhile, is maintained by its binding with link protein and hyaluronic acid, which interacts with the collagen fibrils [159]. It would greatly add to our understanding of the potential utility of MSC micropellets if the expressions of multiple cartilage matrix genes in the differentiated MSCs were analyzed and compared to those of freshly isolated chondrocytes.

One of the limitations of the micropellet study was the lack of protein quantification and in future studies it will be essential to relate mRNA expression in micropellets to protein expression. This will be particularly interesting for the expressions of the collagens investigated here. Translational regulation of collagen I expression has been indicated in chick chondrogenesis, as cartilages not synthesizing detectable amounts of collagen I have been found to contain similar levels of cytoplasmic collagen I mRNA as prechondrogenic mesenchymal cells [252]. Barry et al reported that undifferentiated human BM-MSCs contain collagen X mRNA but not protein and that despite its earlier mRNA increases compared to collagen II mRNA, collagen X protein shows up later than collagen II in differentiated pellets [314]. This has also been reported by Murdoch et al, who found that collagen X mRNA increases simultaneous with collagen II mRNA in their chondrogenic cultures of human BM-MSCs yet regularly observe only faint immunological staining of collagen X in the matrix [317]. In addition to quantification, the distribution of other matrix proteins aside from collagen I and collagen II should be visualized immunologically to more fully characterize the patterns of differentiation.

Select genes which are more prevalent in undifferentiated MSCs, fibrocartilage, hypertrophic chondrocytes, and osteoblasts were also quantified in the initial study. However, a more complete analysis of genes associated with hypertrophy and ossification is still necessary. Some evidence for the suppression of bone markers by MSCs cultured in hypoxic conditions has been provided by others. For example, APase and bone sialoprotein-2 mRNA expressions were found to be reduced during *in vitro* osteogenic induction of human MSCs under 3% O_2 compared to 20% O_2 [372]. Furthermore, temporarily exposing human BM-MSCs to 1% O_2 environment (48 hours) prior to normoxic osteogenic induction resulted in long-lasting downregulation of Runx2/Cbfa1, collagen I, and osteocalcin, although this temporary exposure did not affect APase nor bone sialoprotein-2 expression [378]. The effects of hypoxia on these genes during *in vitro* chondrogenic differentiation may differ from those described during osteogenic differentiation. The low oxygen environment that enhances expression of SOX9, aggrecan, and collagen II in chondrogenic induced micropellets may also alter the expression of other genes generally associated with hypertrophy and ossification. For example, HIF-1α is a transcriptional activator of VEGF and is involved in the regulation of VEGF in mouse limb buds [265]. Additionally, murine chondrocytes cultured

in 0.5% O_2 environment had increased VEGF mRNA expression and protein synthesis compared to those grown under a 20% O_2 environment and deletion of HIF-1α almost completely negated these hypoxic-induced increases [431]. Hypoxia has also been found to increase VEGF expression and secretion by undifferentiated human BM-MSCs *in vitro* [378, 432]. Given the reported propensity for scaffold-free MSC-based chondrogenic aggregates to calcify or degrade in mice ectopic implant models (see Section 2.3.4), investigating the effects of the low oxygen micropellet platform on VEGF and other genes associated with hypertrophy and ossification, such as APase and MMPs, will be essential before moving forward with evaluations of micropellets for tissue engineering applications.

Initially, quantification of gene expression and histological analysis of low oxygen micropellets were performed at only one late time point. The results indicated that differentiation was possibly accelerated compared to conventional pellet cultures. However, future studies should evaluate the progression of changes in gene and protein expression over time, including very early timepoints. This would help to fill in the details as to how BM-MSC chondrogenesis proceeds in micropellets and answer important questions regarding the differentiation process; i.e. whether collagen X expression and synthesis indeed parallels collagen II in this platform or whether it only starts to increase at later timepoints. Such information could provide indications as to how to modify the process with time. Often, as was the case in this study, a chondrogenic medium is formulated and used from the beginning of aggregate formation until the end of the study. It seems unlikely such methods will be capable of producing MSC-based neocartilage comparable in composition and biomechanical properties to permanent articular cartilage. The dynamic signals provided through the developmental stages of chondrogenesis originate from paracrine sources as well as autocrine and changes in environmental factors such as oxygen tension. Temporal changes to medium supplements could be used as a way to better control the process but in order to implement such methods, a detailed time-based characterization of the chondrogenic response must be performed.

6.2.2 Optimization of Parameters for Chondrogenic Induction

In developing the micropellet platform, chondrogenic induction medium was made up using components and associated concentrations that are frequently used for

pellet culture. A review of numerous studies utilizing human BM-MSCs in pellet culture revealed only two commonalities amongst the chondrogenic medium formulations: at least 10 ng/ml of one of the TGF-β isoforms and 10^{-7} M dexamethasone. The first pellet culture studies using rabbit BM-MSCs reported that there was no chondrogenesis in defined medium without either of these factors, rare chondrogenesis when dexamethasone was used without TGF-β, and a dose-dependent chondrogenic response with TGF-β even without dexamethasone [201]. Subsequent studies by this group and others with human BM-MSCs found that pellet cultures treated continuously with only one or the other of these factors resulted in no chondrogenesis and that pellets without TGF-β did not survive for even 14 days [202, 203, 318]. Recently, eight other factors – four BMP isoforms, FGF-1 and FGF-2, insulin-like growth factor-1, and PTHrP – were compared to TGF-β for their ability to induce chondrogenesis in pellet cultures of human BM-MSCs [433]. On their own, in equivalent concentrations, none of these factors aside from TGF-β were reported to stimulate chondrogenesis as assessed by collagen II and proteoglycan deposition. In another recent study, the effect of dexamethasone on chondrogenesis of immortalized human trabecular bone-derived MSCs was investigated, showing that dexamethasone increased collagen XI gene expression within the first 24 hours but that alone it had little effect on aggrecan, collagen II, or COMP expression [434]. After 21 days of co-administration of dexamethasone and TGF-β, mRNA expressions of collagen II and aggrecan were enhanced compared to those of cells treated with only one of the factors; however, immunohistochemical staining did not reveal obvious differences in protein expression amongst the conditions. While a study evaluating the effect of TGF-β withdrawal on chondrogenic induction of human BM-MSC pellets found that sustaining TGF-β exposure increased every measure of chondrogenesis considered (including collagen X expression) [435], published results regarding transient administration of dexamethasone for *in vitro* chondrogenesis are lacking.

In addition to its inclusion in chondrogenic medium, dexamethasone is an important component of osteo-inductive medium; in human BM-MSCs, it is a potent stimulator of APase activity and dose dependently increases mineralization [436, 437]. Recently, the effect of dexamethasone on Runx2/Cbfa1 mRNA expression in human BM-MSCs cultured in standard osteo-inductive medium was reported as a 5-fold increase at 24 hours [438]. Still, as described above, dexamethasone appears to provide

some essential signals for human MSC aggregate chondrogenesis *in vitro*. However, as some effects of dexamethasone on human MSCs are reportedly sustained for weeks following its withdrawal [436, 437], the question of whether continuous treatment with dexamethasone is necessary is one worth investigating. Although continuous dexamethasone treatment increases APase expression and activity and mineralization during *in vitro* MSC osteogenesis, it has been shown to decrease osteocalcin expression in this process and reportedly after one day of dexamethasone treatment this inhibitive effect can last for up to four weeks [436, 438]. Dexamethasone may also have long-lasting effects on genes associated with hypertrophic regulation and this could affect the patterns of gene expression typically observed in MSC chondrogenesis.

PTHrP, an inhibitor of chondrocyte hypertrophy and calcification [276, 277], has been found to increase collagen II and SOX9 expression when added as a late supplement to the chondrogenic medium of pellet cultures of human BM-MSCs and Ad-MSCs [323]. Additionally, in the cultures with PTHrP there were apparent but nonsignificant decreases in Runx2/Cbfa1 and collagen X mRNA expression and less of these proteins and collagen I visualized with immunohistochemistry. In scaffolds seeded with BM-MSCs from OA patients, others have reported that supplementing chondrogenic medium with PTHrP results in decreased collagen X expression and APase activity and unchanged collagen II expression [206]. More recently, Weiss et al found that adding a specific fragment of PTHrP at different timepoints of TGF-β-induced chondrogenesis of human BM-MSCs resulted in decreased collagen X expression and APase activity, as well as decreased collagen II expression [433]. This may be due to the specific fragment used but with the lack of identification of the fragment used in the other studies it is unclear if this is the case or if other differences in study design are responsible for this discrepancy. Taken together, the cumulative evidence indicates that PTHrP reduces hypertrophic and osteogenic marker expression in human BM-MSCs but that there may be context-dependent or fragment-dependent effects on chondrogenic genes. Interestingly, in human BM-MSC monolayer cultures dexamethasone has recently been shown to strongly reduce PTHrP mRNA expression and to suppress endogenous PTHrP release [438]. Following dexamethasone withdrawal, endogenous PTHrP release returned to baseline levels but this recovery took nearly three days. An interesting future study with low oxygen micropellets could evaluate whether brief initial exposure to dexamethasone in this platform is sufficient to promote chondrogenesis and whether

minimizing the period of dexamethasone treatment decreases expression of genes typical of hypertrophy and ossification.

The decision for oxygen levels in this study was based on a review of previous studies investigating the effects of oxygen tension during *in vitro* chondrogenesis. For hypoxic conditions, other researchers have generally chosen from 1-5% oxygen. Here, we tended toward the lower end of this range and 2% O_2 was used as our hypoxic condition. In their scaffold-free Transwell discs of BM-MSCs, Murdoch et al reported that they observed no enhancement of chondrogenesis when they decreased oxygen from 20% to 5% [317]. However, this same group *did* observe increased expressions of the genes for collagen II and aggrecan when human infrapatellar fat pad (IPFP)-derived stem cells were chondrogenically induced in pellet cultures under 5% O_2 compared to pellet cultures under 20% O_2 [364]. While this could be due to differences in cell sources, the potential influence of the geometry of the different systems used in the two studies is an intriguing notion. In their large pellet cultures (500,000 cells/pellet), they note that the oxygen level likely decreases from 5% at the surface of the pellets to somewhat lower in the center. In these pellets, HIF-2α mRNA was increased significantly at 14 days compared to their undifferentiated cells at day 0, whereas HIF-1α mRNA appeared upregulated but not significantly so. Others have found that cultures of human neuroblastoma cells grown under 5% O_2 only had elevated levels of HIF-2α protein while those grown under 1% O_2 had increased levels of both HIF-2α and HIF-1α compared to those grown under 21% O_2 [439]. Therefore, it is possible that the self-induced oxygen gradients in the large pellets of IPFP stem cells contributed to substantially increased HIF-2α and possibly modest increases in HIF-1α, leading to the enhanced chondrogenesis observed in these cultures under 5% O_2. In contrast, a 5% O_2 environment for the thin discs of BM-MSCs in Transwell plates may not have provided as potent a stimulus to affect downstream chondrogenic genes.

Even though a significant increase in HIF-1α in the IPFP stem cell pellets was not seen at 14 days, transient contributions of HIF-1α to increased collagen II and aggrecan expression can not be discounted. In the aforementioned study with neuroblastoma cells, the elevated levels of HIF-1α protein were measured at four hours under 1% O_2 but little to no HIF-1α was detected at three days in these cultures [439]. In another study, monolayer cultures of human ACs were found to have increased collagen II, collagen IX, aggrecan, and SOX9 mRNA expressions after three days grown under 1%

O_2 [369]. Small interfering RNA transfection against HIF-2α significantly decreased the hypoxic-induced expression of collagen II, collagen IX, and SOX9 at three days whereas HIF-1α knockdown had no effect on expression of the collagens but resulted in a lower mean SOX9 expression, although it was declared a nonsignificant change. Interestingly, they later identified the gene that encodes for the beta subunit of activin-A, a TGF-β superfamily member, as being upregulated in human ACs by hypoxia, dependent on both HIF-1α and HIF-2α but independent of SOX9 [371]. In chick mesenchymal limb bud cells *in vitro*, activin-A increases N-CAM expression and enhances chondrogenesis and has been found to be most effective with early administration; activin added to cultures at days one, two, three, and four showed a downward trend in Alcian blue staining for proteoglycans [245]. Recently, human ACs resuspended in alginate beads and cultured under 5% O_2 for 21 days were found to have significantly increased collagen II and aggrecan mRNA levels compared to those cultured under 20% O_2 [370]. Although HIF-1α mRNA expression was not evaluated in these 21-day cultures, cobalt-mediated activation of HIF-1α was shown to lead to substantial increases in collagen II, aggrecan, and SOX9 mRNA levels within 24 hours. Similarly, when the ACs were transfected to overexpress HIF-1α, the relative expression of these genes was found to increase in the first 24 hours and the opposite effect was seen at early timepoints when HIF-1α was inhibited in hypoxic cultures by cadmium-mediated degradation or by transfection with dominant negative HIF-1α.

The results of these studies taken together may explain the discrepancy between the clearly enhanced chondrogenesis of our micropellets differentiated under 2% O_2 and the reported lack of such effects in the thin discs of Murdoch et al differentiated under 5% O_2. Future studies utilizing the micropellet system could explore the effects of varying the oxygen levels incrementally between the extremes presented here. The very small size of aggregates in this system should allow exposure of the differentiating cells to a narrow range of oxygen and thus provide further insight into the effects of different levels of oxygen tension on MSCs, specifically the contributions of HIF-1α and HIF-2α with time and as oxygen levels change. This could answer fundamental questions about how oxygen exerts its effects on this process as well as aid us to optimize the parameters for chondrogenic differentiation in micropellets or other platforms.

6.2.3 Considerations for Future Applications

The micropellet system was designed not only to enhance chondrogenesis but to do so in a way that is compatible with integration with current tissue engineering strategies. This compatibility is aided by the nonadhesive substrate as the micropellets can easily be differentiated for an appropriate period of time and then collected by simply rinsing the wells to push the aggregates out of the microwells. They could then be used in a manner similar to how a cell suspension of chondrocytes could be used. For example, in ACI a suspension of chondrocytes is injected under a flap of periosteal tissue or a collagen patch. Following further optimization, studies evaluating the utility of micropellets for ACI-type interventions should be undertaken in animal models. Correspondingly, it would be important to evaluate the effects of the platform on redifferentiation of ACs and how micropellets of ACs, micropellets of MSCs, and single cell suspensions of ACs or MSCs compare in such an intervention. While the availability of MSCs as a cell source is a distinct advantage compared to ACs, their clinical usefulness ultimately depends on achieving at least equivalent outcomes as ACI already provides.

Another possible application of the micropellet platform is to create scaffold-free neocartilage tissues by collecting micropellets into larger masses and further culturing them prior to implantation. The aforementioned studies to better characterize the temporal response of micropellets would guide these efforts by providing indications for when micropellets should be pooled. One aspect of the low oxygen micropellets that is very promising is the extremely high amount of proteoglycans that are produced. Unfortunately, as is often the case with pellet cultures, the majority of the proteoglycans is lost to the supernatant and not retained within the matrix of the differentiating aggregates. A membrane bioreactor we developed using polydimethylsiloxane (PDMS), Cuprophan™ (10 kDa molecular weight cut-off cellulose membrane), and glass cylindrical reservoirs has been considered as a potential solution (Fig. 6.2) [440]. This bioreactor prototype was originally designed by Dr. Michael Doran at The University of Queensland to aid in HSC expansion but we also found that pellet cultures of human BM-MSCs (2% O_2 cultures, as described in Section 4.4.2) that were transferred to the bioreactors after three days would continue producing sGAGs at similar levels as pellet cultures transferred to bioreactors without membranes (Fig. 6.3). However, in membrane bioreactors all of the sGAGs were retained in the matrix of the cell aggregates while in

the bioreactors without membranes the pellets lost more than half of their totals to the supernatant. The membrane between the bulk medium and the well where cells are maintained prevents the escape of proteoglycans and other large molecules synthesized by the cells and these results indicate that proteoglycan production is not negatively impacted by the bioreactor membrane. Additionally, these cultures were maintained with only the high local concentration of TGF-β added prior to putting the bioreactors together. Medium changes to bioreactors without membranes included the normal refreshing of TGF-β every three to four days but medium changes to the bulk reservoir of membrane bioreactors did not include TGF-β as it is too large to pass through the membrane. While these results only provide a glimpse of the possibilities, pooling micropellets at an appropriate time and transferring them to a bioreactor such as this may allow for the retention of a larger quantity of proteoglycans than is typically achieved, as well as saving on expensive growth factors.

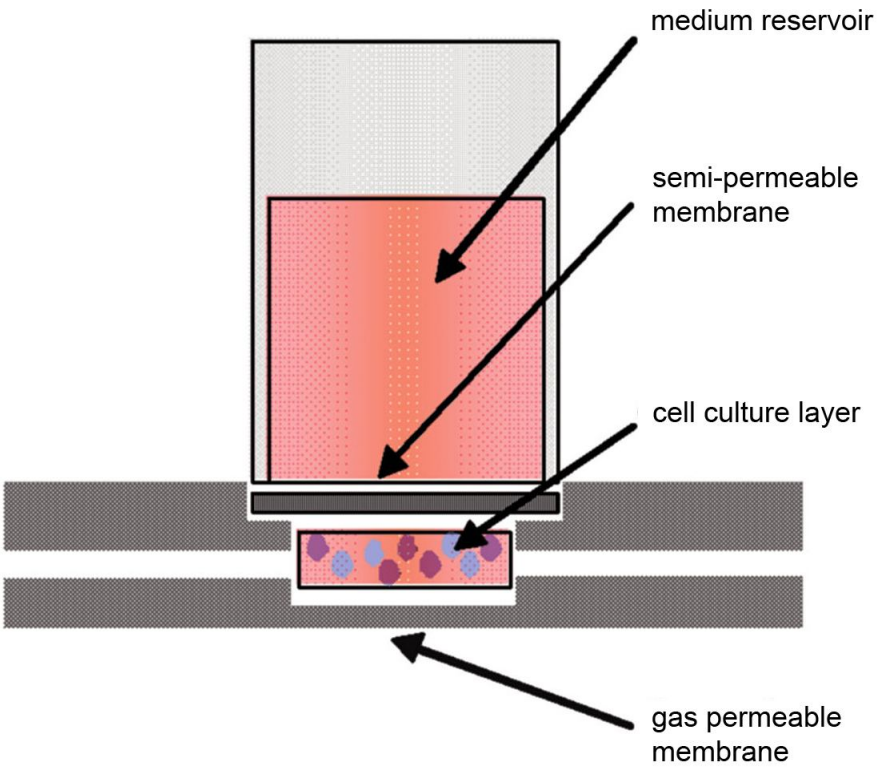

Figure 6.2. Schematic of membrane microbioreactor. Briefly, construction of the microbioreactor consists of the following steps. Cuprophan membrane is fixed to a glass cylinder using silicone sealant to make the upper medium reservoir. Holes (6-mm diameter) are punched out of 300-µm PDMS film spun coated onto an overhead transparency. The medium reservoir is aligned with the 6-mm hole in the PDMS film and fixed in place with silicone sealant. To further secure the medium reservoir, PDMS is cast around the outside of the reservoir. The reservoir is removed from the overhead transparency and aligned with the base membrane and microwell (formed by spin casting PDMS over SU-8 photoresist microwell feature on a silica wafer).[3]

[3] This research was originally published in *Tissue Engineering Part C: Methods*. Doran, M.R., Markway, B.D., Clark, A., Athanasas-Platsis, S., Brooke, G., Atkinson, K., Nielsen, L.K.., and Cooper-White, J.J., "Membrane Bioreactors Enhance Microenvironmental Conditioning and Tissue Development," *Tissue Eng Part C Methods*. © 2009 Mary Ann Liebert, Inc.

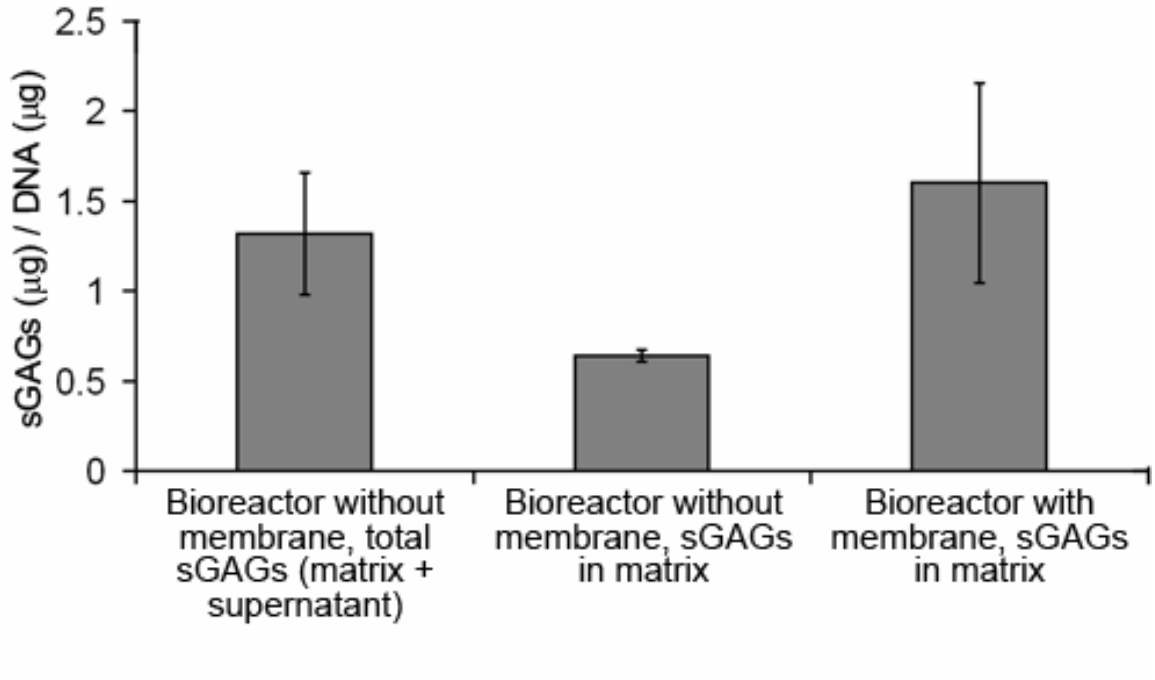

Figure 6.3. Sulfated GAG production by chondrogenic cultures of human BM-MSCs in membrane microbioreactors. Pellet cultures transferred to membrane bioreactors produced similar levels of total sGAGs/DNA over 14 days as cultures in bioreactors without membranes. For the membrane bioreactors, no sGAGs were detectable in the supernatant collected from the bulk medium at days 3, 6, 10, and 14. For bioreactors without membranes, the total amount of sGAGs is the cumulative amount from supernatant measured at days 3, 6, 10, and 14 in addition to that measured within the digested cell matrix. Values are mean ± SD from N=4 bioreactors with membranes and N=4 without.[3]

These are just two potential ways to utilize micropellets for tissue engineering interventions. There is much characterization and optimization still required to arrive at a point where models for tissue engineering applications can be legitimately initiated. However, it is believed that these initial studies show their promise with regard to finely controlling chondrogenesis and that with improved methodologies, the properties of low oxygen micropellets will make them candidates for integration with the proposed systems as well as scaffold-based cartilage repair strategies.

[3] This research was originally published in *Tissue Engineering Part C: Methods*. Doran, M.R., Markway, B.D., Clark, A., Athanasas-Platsis, S., Brooke, G., Atkinson, K., Nielsen, L.K.., and Cooper-White, J.J., "Membrane Bioreactors Enhance Microenvironmental Conditioning and Tissue Development," *Tissue Eng Part C Methods*. © 2009 Mary Ann Liebert, Inc.

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Biographical Sketch

Brandon Davis Markway was born in Columbia, Missouri on September 30, 1980 to Albert and Debbie Markway. He grew up in Clinton, Missouri and following high school he attended the University of Missouri – Columbia (Mizzou) to study chemical engineering. A brief stint at the University of Arizona for National Student Exchange during his second year of undergraduate education would have otherwise been academically forgettable had he not discovered that he enjoyed biology more than he'd previously thought. Upon returning to Mizzou, Brandon focused on biochemical engineering and later earned a National Science Foundation research placement at Boston University for a summer. After another term at Mizzou, Brandon undertook a six month co-op experience at ALZA Corporation in drug delivery R&D. He then returned to Mizzou and graduated Magna Cum Laude with a B.S. in Chemical Engineering in 2004 before attending Oregon Health & Science University for graduate school that fall.

Brandon's graduate work initially focused on investigating surfaces for vascular engineering strategies to utilize circulating progenitor cells. In 2008, he was awarded a Whitaker International Fellowship to spend a year at The University of Queensland in Brisbane, Australia. There his interest in stem cell-based cartilage engineering developed and his productive efforts in that work led to an interesting experience compiling his dissertation. Overall, from his time as a graduate student Brandon produced two first-author papers, was a contributing author to four others, and has presented his work at four conferences in four different countries. He hopes to continue this last trend. His published and presented work includes:

PUBLICATIONS

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ABSTRACTS

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