Multilineage Differentiation of Mesenchymal Stem Cells for Cell-based Tissue Engineering

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THESIS

Submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in Biopharmaceutical Sciences in the Graduate College of the
University of Illinois at Chicago, 2014

Chicago, Illinois

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Dedicated to my family Gabriele, Matthias and Patrick Koellmer.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ADIPQ	Adiponectin
ACAN	Aggrecan
ALPL	Alkaline phosphatase
APS	Ammonium persulfate
BCL-2	B-cell lymphoma 2
BGLAP	Bone Gamma-Carboxyglutamate (Osteocalcin)
BM	Bone marrow
BMP	Bone morphogenetic protein
CAM	Chick chorioallantoic membrane
CD309	Vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR)
CFE	Colony forming efficiency
CFU	Colony forming unit
Col	Collagen
DDIW	Double deionized water
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGM-2	Endothelial growth medium-2
EMSC	Endothelial-like hMSC
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell

ePFTE	Expanded polytetrafluorethylene
ESC	Embryonic stem cell
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H&E	Hematoxylin & eosin
hMSC	Human mesenchymal stem cell
HUVEC	Human umbilical vein endothelial cell
FABP4	Fatty acid-binding protein-4
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLT-1	Fms-Related Tyrosine Kinase 1 (VEGF-R1)
FN	Fibronectin
HSC	Hematopoietic stem cell
IL	Interleukin
KDR	Kinase-insert domain containing receptor (VEGF-R2, CD309)
LAMA	Laminin
LEP	Leptin
LPL	Lipoprotein lipase
MSC	Mesenchymal stem cell
MMP	Matrix metalloproteinase
MHC	Major histocompatibility complex
OC	Osteocalcin
ON	Osteonectin
OPN	Osteoopontin
PC	Pericyte
PCL	Poly(caprolactone)
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor

PEGDA	Poly(ethylene glycol) diacrylate
PLA	Poly (D,L-lactide)
PLGA	Poly(lactide-co-glycolide)
PPARγ2	Peroxisome proliferation-activated receptor γ2
RGD	Arg-Gly-Asp peptide
RPL13α	Ribosomal protein L13α
RT	Room temperature
Runx	Runt-related transcription factor
SMC	Smooth muscle cell
SPH	Superporous hydrogel
SPH-Col	SPH with collagen embedded into the pores of the scaffold
SPH-Col-EMSCs	SPHs that were precultured with collagen-embedded EMSCs
SPH-Col-hMSCs	SPHs that were precultured with collagen-embedded hMSCs
SPH-Col-HUVECs	SPHs that were precultured with collagen-embedded HUVECs
STRO-1	Stromal cell surface antigen
TCP	Tissue culture plastic
TE	Tissue engineering
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor receptor 2
VE-cadherin	Vascular endothelial-cadherin
vWF	von Willebrand Factor

SUMMARY

Human mesenchymal stem cells (hMSCs) derived from adult tissues have shown increasing potential in regenerative medicine due to their multilineage differentiation capability and their immunomodulatory effects. Current tissue engineering (TE) approaches typically involve the development of stem cell-carrying extracellular matrix (ECM)-mimicking scaffolds that mature *in vitro* before implantation into the patient. To control for lineage specific differentiation, ideal scaffold materials should be able to maintain stem cells in their undifferentiated phenotype and to promote differentiation only after induction.

In the first part of this dissertation, we evaluated the utility of poly(ethylene glycol) diacrylate (PEGDA) superporous hydrogel scaffolds (SPH) for stem cell delivery in tissue engineering (TE) applications. Generally, flat PEGDA surfaces do not promote protein adsorption and are thought to be devoid of cell-matrix interactions. We observed a different scenario in the SPHs and showed for the first time that unmodified PEGDA SPHs can provide a microenvironment that enables stem cell-derived extracellular matrix (ECM) development and thus promotes stem cell survival. We believe that architecture and the physicochemical characteristics - interconnected pores ranging from 100 to 600 μm - of the scaffold hold properties that foster cellular responses. We detected collagen type I, collagen type IV, fibronectin and laminin on mRNA and protein levels within hMSC-seeded PEGDA SPHs. The cell-secreted ECM resembles the composition of the *in vivo* stem cell niche, the bone marrow. After long-term culture within PEGDA SPHs, hMSCs expressed stem cell surface markers, CD105, CD90, CD73 and CD44, to a similar extent than their counterparts grown on 2D monolayer suggesting that the cells preserved their stem cell phenotype.

SUMMARY (continued)

To investigate whether hMSCs also remain functional within the SPHs, we cultured the cells for three weeks in basal medium before inducing the differentiation process for four weeks. Tissue-specific histological staining and the expression of adipogenic, chondrogenic, and osteogenic marker genes in the appropriate lineage demonstrated that hMSCs are able to undergo controlled multilineage differentiation within PEGDA SPHs. Thus, we demonstrated that PEGDA SPHs provide an opportunity for culture of viable and functional hMSCs under controlled conditions for stem cell maintenance and differentiation.

Generally, a small subset of marker genes is used to evaluate stem cell differentiation towards a certain lineage within TE constructs. Currently, there is a lack of studies looking at the possibility for expression of these markers in multiple lineages. Due to the heterogeneous nature of hMSCs and the known plasticity between adipogenic and osteogenic lineages (Schilling et al., 2007), we assessed adipocyte- and osteoblast-associated genes for their suitability to indicate differentiation towards the respective lineage. We identified fatty acid binding protein P4 (FABP4) as specific adipogenic gene marker. All osteogenic marker genes (alkaline phosphatase, collagen type I, osteopontin, osteocalcin) tested were also expressed in adipocyte-derived hMSCs. With alkaline phosphatase and osteopontin in particular being upregulated during adipogenic differentiation. The work in this dissertation clearly indicates that many of the markers used for determining the end fate are shared between adipogenic and osteogenic differentiated hMSCs. Thus, more definitive markers are needed to clearly eludicate the differentiation status of the cells, and differentiation towards a certain lineage should be accompanied by morphological and histological observations.

SUMMARY (continued)

Besides the proper identification of the differentiated tissue it is also important to keep it alive. A major challenge in TE lies in ensuring the survival of engrafted cells within TE constructs after *in vivo* implantation. Recent advances in prevascularization strategies suggest that forming capillary structures within scaffold materials *in vitro* enhances anastomosis (forming connections) with the ingrowing host vessels after implantation and thus leads to a faster supply of blood and oxygen. In this dissertation, we further assessed the endothelial differentiation potential of hMSCs with the goal of using the differentiated cells for the prevascularization of PEGDA SPHs. Our results indicate that bone marrow-derived hMSCs acquire several endothelial-like characteristics when cultured in endothelial growth medium that was supplemented with additional VEGF. When cells were induced directly within a collagen-filled SPH, capillary-like structures could be observed after 14 days of endothelial differentiation. SPHs that were prevascularized with endothelial-like MSCs (EMSCs) exhibited no inflammatory response when implanted onto the chick chorioallantoic membrane (CAM) for 7 days. Blood vessel infiltration was seen to a similar extent in cell free and prevascularized SPHs but mature, collagen-surrounded blood vessels were only present in SPHs that were preseeded with EMSCs or the standard endothelial cell line (HUVECs). Our data suggest a potential benefit of MSC-derived ECs for prevascularization but further studies are warranted to elucidate the stability and functionality of the *in vitro* formed capillary-like structures.

In this dissertation, we identified a suitable scaffold for stem cell delivery, demonstrated the need for more specific differentiation markers and showed the potential of hMSCs in

vascularization approaches. The conclusions obtained from this dissertation will set the ground for future investigations, at both a fundamental level and from a TE standpoint.

1 Introduction

1.1 Cell-based tissue engineering

Langer and Vacanti defined tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer and Vacanti, 1993). In contrast to many pharmacological therapies, successful tissue engineering (TE) approaches could provide lasting solutions to the problem of organ failure. Generally, TE exploits three main approaches (*i*) the use of cells to replace cellular components, (*ii*) the use of acellular scaffolds, often with incorporated growth factors, capable of inducing tissue regeneration or (iii) a combination of both (Khademhosseini et al., 2006). The last approach is known as cell-based TE.

Utilizing cells that release soluble factors in biomaterials might overcome the problems associated with growth factor delivery. Most, if not all, of the current TE systems in which growth factors are used result in a burst release of the growth factor shortly after placement. In some cases this can have adverse effects on tissue regeneration. Ozawa *et al*. showed that high vascular endothelial growth factor (VEGF) concentrations have detrimental effects on vessel growth and can promote hemangiomas (Ozawa et al., 2004).

The persistence of debilitating diseases like cancer and arthritis and the shortcomings of conventional grafting methods show a growing need for new tissues and tissue-like materials. However, the successful engineering of functional tissue still has to overcome many obstacles, such as the identification of appropriate cell sources and the creation of artificial microenvironments that allow for optimal cell function. The discovery and implementation of stem cells opened up the potential of developing stem cell-based tissue engineering

applications. Mesenchymal stem cells (MSCs) are a source of large numbers of autologous cells with a great differentiation capacity. Three-dimensional scaffolds and bioreactor cultures have been shown to provide suitable microenvironments for tissue formation with mesenchymal stem cells (MSCs) (Burdick and Vunjak-Novakovic, 2009).

1.2 Human mesenchymal stem cells in cell-based tissue engineering

1.2.1 Identity and function

Mesenchymal stem cells (MSCs) are a source of stem/progenitor cells that were first identified as colony forming unit (CFU's) fibroblast like cells of the bone marrow by Friedenstein in the 1970s (Friedenstein et al., 1974). Their incidence among other bone marrow cells was estimated to be 0.001% to 0.01% (Friedenstein et al., 1974; Penfornis and Pochampally, 2011). MSCs are also referred to as multipotent mesenchymal stromal cells because they belong to the stroma that is believed to provide a supportive environment to the hematopoietic stem cell (HSC) niche (Nombela-Arrieta et al., 2011). Recent studies showed that MSCs can also be isolated from other tissues, including adipose tissue, placenta, trabecular bone, femur, skeletal muscle and dental pulp (Cowan et al., 2004; Miao et al., 2006). MSCs are plastic adherent and can be expanded in culture while maintaining their two most important features, the ability to differentiate into multiple mature cell types (multipotency) and the ability to self-renew (Nombela-Arrieta et al., 2011). When cultured on tissue culture plastic (TCP) MSCs maintain a normal karyotype and normal telomerase activity at least up to passage 12 (Pittenger et al., 1999). To facilitate the comparison of results across studies, the International Society for Cytotherapy proposed minimal criteria to define MSCs, including (1) adherence to plastic in culture, (2) the expression of CD105, CD73, and CD90 in greater than 95% of the culture, and their lack of expression ($\leq$ 2%) of

either "CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II", and (3) the ability to differentiate into adipocytes, chondrocytes and osteoblasts *in vitro* (Dominici et al., 2006).

In addition to their multilineage differentiation potential, MSCs have been shown to control immune and inflammatory responses (Maxson et al., 2012; Wu et al., 2007). MSCs directly attenuate inflammation by tumor necrosis factor-α (TNF-α) suppression, interleukin (IL)-10 and IL-4 production as well as T-cell proliferation blockade (Maxson et al., 2012). Due to these functions MSCs are beneficial in wound repair. MSCs, that were applied on the surface of deep burn wounds, decreased the infiltration of inflammatory cells into the wounds, accelerated angiogenesis and formation of granulation tissue (Shumakov et al., 2003). Further, MSCs have been shown to delay skin graft rejection (Bartholomew et al., 2002) and to facilitate the engraftment of hematopoietic stem cells by alleviating graft versus host disease (Lazarus et al., 2005) MSCs often lack expression of major histocompatibility complex (MHC) II and costimulatory molecules. Thus, besides the possibility to use the patient's own cells, allogenic cells could be implanted into immunocompetent patients (Aggarwal and Pittenger, 2005). MSCs avoid allogenic rejection possibly due to their hypoimmunogenic properties, their ability to modulate T cells and to create an immunosuppressive local environment (Ryan et al., 2005). MSCs preferentially home to sites of injury and induce repair, either by differentiating into the cell type of need or by creating an environment that increases the capacity of local cells to repair tissue (Prockop, 2009). MSCs secrete a variety of growth factors and chemokines, especially vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF), and also contribute to tissue repair via

paracrine signaling (Maxson et al., 2012). Thus, MSCs are an attractive cell source in cell-based TE due to availability, their capacity to differentiate into multiple cell types, their immunomodulatory properties and their implications in wound healing.

1.2.2 Isolation of MSCs

MSCs are generally isolated from bone marrow aspirates harvested from the iliac crest in humans (Pittenger et al., 1999). Frequently, the whole bone marrow sample is fractionated on a density gradient solution such as Percoll®, after which the cells are plated on tissue culture plastic. Primary cultures are usually maintained for ~ two weeks to allow time for the depletion of non-adherent haematopoietic cells. The property of plastic adherence is often not sufficient for the purification of MSCs and heterogeneous cell populations with varying proliferation and differentiation potentials were observed (Pittenger et al., 1999). Pittenger *et al.* reported that out of six colonies derived from clonal cells of bone marrow-derived MSCs, all could be induced towards the osteogenic lineage but only 5 underwent adipogenic differentiation, and only two underwent chondrogenic differentiation (Pittenger et al., 1999). It was suggested that the *in vitro* culture conditions may have caused some loss of multipotency or that some cells might represent progenitor cells, with restricted differentiation potential. The later hypothesis is currently challenged by the proposed plasticity between committed progenitors (Schilling et al., 2007; Song and Tuan, 2004). Nevertheless, to select for specific cell populations, immunophenotyping with flow cytometry is necessary. Besides the markers proposed by the International Society for Cytotherapy, MSCs generally express STRO-1 (a stromal cell surface antigen), CD44 (hyaluronate), CD106, CD120a, CD124 and CD166 (Kolf et al., 2007; Pittenger et al., 1999). However, until now no explicit marker that can distinguish MSCs from other cell types is

known and the surface marker profile might vary in MSCs from different origins and species. For instance, CD34 is expressed in MSCs derived from mice, but not in human MSCs (Copland et al., 2008). Although bone marrow derived MSCs are still the most frequently investigated cell type, MSCs isolated from adipose tissue (Fraser et al., 2006), peripheral blood (Wan et al., 2006), the placenta (Barlow et al., 2008), the lung (Griffiths et al., 2005) or the heart (Beltrami et al., 2003) have also shown potential for the differentiation into different cell types (Hass et al., 2011). However, MSCs from different tissues exhibit diverse proliferation rates, gene expression profiles and differentiation potential (Hass et al., 2011; Shetty et al., 2010; Strioga et al., 2012). Further, the amounts of MSCs that can be obtained from different tissues also vary. Only 0.001 to 0.01% of the cells isolated from bone marrow aspirates by density gradient centrifugation represent MSCs (Pittenger et al., 1999). From 1 g adipose tissue, 5×10^3 MSCs can be obtained, which is significantly more than from an equivalent amount of bone marrow (Fraser et al., 2006; Hass et al., 2011). Out of one million peripheral blood-derived mononuclear cells, only 1.2 to 13 cells exhibit colony forming efficiency (CFE) (Hass et al., 2011; Wan et al., 2006).

1.2.3 Origin of MSCs - *in vivo* niche

MSCs can be obtained from a variety of tissues raising the question if there is a common niche environment in all these tissues. The concept of a stem cell niche was first introduced by Schofield in 1978 who describes it as an environment where the "stem cell is seen in association with other cells which determine its behavior. It becomes essentially a fixed tissue cell. Its maturation is prevented and, as a result, its continued proliferation as a stem cell is assured" (Schofield, 1978). The niche encompasses all elements that surround the stem cells when they are in their undifferentiated naïve state, including other cell types,

extracellular matrix (ECM) and local soluble molecules (Kolf et al., 2007). All of these factors act together to promote stem cell maintenance and self-renewal. It is further assumed that other cues must find their way into the niche to induce differentiation needed for tissue regeneration.

MSCs were found to line blood vessels in their tissue of origin and thus a perivascular nature of the MSC niche has been suggested (Shi and Gronthos, 2003). The microvasculature as niche location would permit MSCs access to all tissues. This would further contribute to the hypothesis that MSCs are integral in the healing process of many tissues. The bone marrow (BM), currently the most studied niche environment, promotes stem cell renewal or the differentiation of MSCs into osteoblasts, adipocytes and fibroblastic reticular cells to provide a supportive environment for hematopoietic stem cells (HSCs) (Nombela-Arrieta et al., 2011). MSC-derived osteoblasts are thought to regulate HSC homeostasis and play important roles in many stages of the hematopoietic development (Garrett and Emerson, 2009). On the other hand, MSC-derived adipocytes negatively regulate hematopoietic progenitors (Naveiras et al., 2009).

1.2.4 Multilineage differentiation of MSCs

MSCs have been shown to differentiate towards the osteogenic lineage after induction with dexamethasone, β-glycerol phosphate, and ascorbic acid (Pittenger et al., 1999; Vater et al., 2011a). Calcium accumulation and an incease in alkaline phosphatase (ALP) activity define the osteogenic morphology. The mineralized matrix can be visualized with alizarin red staining, which reacts with the calcium ions within the mineralized matrix (Lievremont et al., 1982). Von Kossa is another staining method and is based on the substitution of phosphate bound-tissue calcium by silver ions (Sheehan and Hrapchak, 1980). Runt-related

transcription factor-2 (Runx-2), ALP, collagen type I, osteonectin (ON) and bone morphogenetic protein-2 (BMP-2) are considered to be early gene markers of osteogenic differentiation, whereas osteocalcin (OC) and osteopontin (OPN) are present at later stages of bone development (Long, 2001; Zhu et al., 2001). The osteogenic potential of MSCs is conserved through numerous passages and thought to be the last one to extinct (Pittenger et al., 1999).

Chondrogenic differentiation of MSCs can be induced in dexamethasone- and transforming growth factor (TGF)-β3-containing medium (Pittenger et al., 1999; Vater et al., 2011a). Chondrogenic differentiation is further promoted when the isolated MSCs are centrifuged to form a pelleted micromass. Close cellular contacts result in the formation of cadherin- and connexin-mediated adhesion complexes required for mesenchymal condensation and the deposition of cartilage (Tuli et al., 2003; Zhang et al., 2002). The chondrogenic phenotype is characterized by the secretion of anionic proteoglycans and extracellular matrix mainly type II collagen and aggrecan. The proteoglycans stain positive with safranin O, toluidine blue, alcian blue (Shepard and Mitchell, 1976; Vater et al., 2011a). Sex determining region (SRY)-related high-mobility group box 9 (Sox9) is an early transcription factor in the chondrogenic differentiation process that controls gene expression of collagen type 2 (Col2), collagen type 9 (Col9), collagen type 10 (Col10), collagen type 11 (Col11), aggrecan and cartilage link protein (Vater et al., 2011a).

To induce differentiation into the adipogenic lineage, MSCs are cultured in the presence of dexamethasone, insulin, indomethacin, and 1-methyl-3-isobutylxanthine (Pittenger et al., 1999; Vater et al., 2011a). The adipogenic phenotype is characterized by the accumulation of lipid-loaded vacuoles within cells that continue to develop over time, coalesce and eventually

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fill out the cells. Lipophilic dyes like Oil red O and Sudan III stain lipid vacuoles bright red. MSC-derived adipocytes have been shown to remain healthy in culture for at least 3 months (Pittenger et al., 1999). MSC-derived adipocytes express peroxisome proliferation-activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), and the fatty acid-binding protein-4 (FABP4/aP2) (Pittenger et al., 1999). FABP4 and the adipokines, adiponectin and leptin, are considered as late adipogenic differentiation markers (Vater et al., 2011a).

1.2.5 Plasticity of MSCs

In contrast to pluripotent embryonic stem cells (ESCs), which can be induced into all cell types of the adult body, adult mesenchymal stem cells appeared to be more restricted in their differentiation potential and to be more committed to tissues of mesodermal origin. MSCs were initially described as fibroblastoid cells with the capability to differentiate into mesodermal cell types, including osteocytes, chondrocytes, and adipocytes and to form osseous tissue when implanted *in vivo* (Dominici et al., 2006). However, new findings oppose the central dogmas of commitment of MSCs by demonstrating their transdifferentiation potential along other germ layers and even within the mesenchymal system. Although still contradictory, several research groups reported on the potential of bone marrow-derived MSCs to differentiate into functional neurons (Chen et al., 2001; Tondreau et al., 2008). Following exposure to reducing agents and antioxidants, MSCs adopted a neuron-like morphology and expressed various neural specific proteins including nestin, glial fibrillary acidic protein, neurofilament heavy chain and *β*-III tubulin (Sanchez-Ramos, 2002). MSCs also appear to be involved in the generation of myocardial cells (Choi et al., 2011), endothelial-like cells (Oswald et al., 2004), and hepatocyte-like cells (Christ and Dollinger, 2011). However, it is still uncertain whether "true" progenitor cells from non-

mesodermal origin can be obtained from MSCs. In *in vivo* experiments these cells often fail to generate the respective tissue (Mohamadnejad et al., 2010).

Transdifferentiation was also observed between mesodermal lineages. Song and Tuan showed that fully differentiated bone marrow-derived osteoblasts were able to change their differentiation agenda to become adipocytes and chondrocytes after induction with the respective differentiation media (Song and Tuan, 2004). In the same way, differentiated chondrocytes could be differentiated into osteoblasts and adipocytes (Song and Tuan, 2004). The authors suggested that committed progenitors, comparable to primary cells, can dedifferentiate and return to a stem cell-like stage when the inducing factors are removed.

Due to the overlap of medium components (dexamethasone) within differentiation protocols and the proposed plasticity between mesodermal lineages, it cannot be excluded that markers are expressed by multiple cell types when MSCs are differentiated using common differentiation protocols. The *in vitro* heterogeneity of hMSCs as a result of the presence of different progenitor stages, for example, cells of the osteoblastic or adipocytic lineage could also contribute to unspecific marker expression. However, proper characterization of hMSCs is crucial for clinical application. Thus, chapter 3 of this thesis examines the specificity of adipogenic and osteogenic differentiation markers.

1.2.6 Mesenchymal stem cells in vascular TE

1.2.6.1 The need for an alternative endothelial cell source

A limitation of current TE technologies is the availability of an expandable, autologous endothelial cell source. Although endothelial cells (ECs) have been used in numerous TE applications their clinical applicability is limited due to the complicated (invasive) harvesting procedure, their low proliferation rate *in vitro* and their phenotypic heterogeneity. Cells that

act as endothelial cells are needed to create capillary-like structures in TE scaffolds or to line artificial vessels to restore vascularization in vascular grafts. Embryonic stem cells (ESCs) have been used to create endothelial-like cells (Hatano et al., 2013). However, teratoma formation has been observed after transplantation of embryonic stem cell seeded scaffolds into severe combined immunodeficient mice (Lees et al., 2007). MSCs could serve as an alternative cell source to primary endothelial cells and ESCs. Considering the plasticity of MSCs it is hoped that functional endothelial cells can be produced.

The potential benefit of MSCs for vascular tissue engineering, whether as endothelial or perivascular progenitors, has been proposed by several studies (Huang and Li, 2008). MSCs release angiogenic growth factors and could promote vessel formation and wound healing by paracrine mechanisms (Chen et al., 2008; Mayer et al., 2005). Co-transplantation of MSCs with hematopoietic stem/progenitor cells (HSCs) resulted in augmented *in vivo* vascularization of a TE scaffold (Moioli et al., 2008). MSCs further acted as perivascular support cells that expressed smooth muscle markers and engineered blood vessels derived from HUVECs and MSCs remained functional for 130 days *in vivo* (Au et al., 2008). MSCs have also been shown to acquire a pericyte-like $CD146^+$ phenotype by supplementation of the basal growth medium with TGF-ß1 (Mendes et al., 2012).

1.2.6.2 Endothelial differentiation of MSCs

Several studies have demonstrated the endothelial differentiation potential of MSCs *in vitro* (**Table 1**). Differentiation was evaluated by the expression of specific endothelial markers or by functional assays. Typical endothelial cell markers include VEGF receptors 1 and 2 (Flt-1 and KDR), vascular endothelial (VE)-cadherin, platelet-endothelial cell adhesion molecule 1 (CD31), CD34, von Willebrand Factor (vWF), and vascular cell adhesion molecule-1

(VCAM-1) (Oswald et al., 2004). To test for EC function, tube formation assays on Matrigel and the uptake of acetylated low-density lipoproteins are conducted.

Differentiated endothelial-like MSCs have not only been shown to generate functional blood vessels *in vivo* but also to perform better than undifferentiated MSCs with respect to neovascularization (Janeczek Portalska et al., 2012; Liu et al., 2007). Since MSCs are already used in TE, MSC-derived ECs would be an ideal cell source for the prevascularization of TE constructs. Yet, no universal endothelial differentiation protocols for MSCs have been established and studies with various outcomes have been conducted (**Table 1**).

Only few reports investigated the use of MSC-derived endothelial cells in TE applications (Janeczek Portalska et al., 2012; Liu et al., 2013; Portalska et al., 2013a; Sahar et al., 2012). Janeczek Portalska *et al.* showed that prevascularization of PLA scaffolds with human endothelial-like MSCs led to a greater angiogenic response *in vivo* than prevascularization with MSCs and HUVECs (Janeczek Portalska et al., 2012). Sahar *et al.* compared *in vivo* vascularization and bone formation of poly (D,L-lactide) (PLA) scaffolds that were either seeded with endothelial-like adipose-derived stem cells, MSCs that underwent osteogenic differentiation or undifferentiated MSCs prior to implantation into mice for 8 weeks (Sahar et al., 2012). Scaffolds containing endothelial-like MSCs exhibited a higher (although not statistically significant) microvessel density but bone formation was significantly reduced compared to the other cell-seeded constructs. However, another study demonstrated that scaffolds containing co-cultures of endothelial-like rabbit-derived stem cells and MSCs displayed a higher amount of regenerated bone compared to MSC-seeded scaffolds only when implanted into bony defects of the rabbit mandible (Liu et al., 2013).

In contrast to blood-derived endothelial progenitor cells (EPCs), MSCs can be isolated in greater number from adult sources and possess a better expansion capacity *ex vivo* (Schatteman et al., 2007). However, when using MSCs for obtaining endothelial-like cells, one has to consider the possibility of varying endothelial differentiation efficiencies between different stem cell origins and among donors.

In Chapter 4 we assessed the *in vitro* differentiation potential of bone marrow-derived hMSCs towards the endothelial lineage. It is our belief that MSC-derived endothelial-like cells can be used to enhance *in vivo* angiogenesis and vascularization of a tissue engineering scaffold.

1.2.7 Translation of stem cell-based therapies

At the time of writing this thesis, the open access clinical trial database http://clinicaltrials.gov showed 359 trials using MSCs for a variety of therapeutic applications. Among other therapies, MSCs are being tested for the treatment of bone defects, acute myocardial infarction, multiple sclerosis, leukemia, diabetes, chronic wounds, and in graft-versus-host disease. There are currently 11 clinical trials that combine MSCs with scaffolds for bone and cartilage repair. In 2012 the first stem cell-based drug from Osiris Therapeutics got approved in Canada. Prochymal is prepared with mesenchymal stem cells to treat people whose own bone marrow is depleted due to chemo- or radiation therapy. In the US, Prochymal is being evaluated in Phase 3 clinical trials for acute graft versus host disease (GvHD) and Crohn's disease. However, despite the extensive research going on in this area, there are no FDA approved MSC-based tissue engineered products on the market yet.

For clinical translation, some fundamental questions about MSCs still have to be answered. Future studies should be directed to explore more definitive markers for identification and to

understand the endogenous role of MSCs in normal and diseased tissue. Further, effective and safe MSC doses for cell injections have to be determined. Also, alternative ways of using MSCs should be explored. For instance, identification of the factors that contribute to the anti-inflammatory and immunomodulatory effects of MSCs would help to create drugs based on these factors. This approach might overcome the complex regulatory requirements that delay clinical implementation of stem cell therapy. A prerequisite for using MSCs in TE applications is to develop biomaterials and scaffolds that allow for cell viability and controlled differentiation.

1.3 Biomaterials as structural frameworks for mesenchymal stem cells

1.3.1   Recreating the stem cell niche *in vitro*

Cells cannot form complex tissues by themselve, therefore, a template or a scaffold is needed to provide control over tissue architecture and mechanical properties. The establishment of niche environments *ex vivo* would be of great benefit in the understanding of the mechanisms involved in MSC regulation and also for creating functional stem cell-based TE constructs. Three-dimensional platforms such as TE scaffolds provide complex environments that have been shown to better mimic the *in vivo* milieu of cells than their two dimensional counterparts (Burdick and Vunjak-Novakovic, 2009). Advanced materials that contain adhesive ligands, cytokines, and growth factors have been designed to control the fate of incorporated cells (Edalat et al., 2012; Papavasiliou et al., 2010). The surface and morphology of these materials function as platform for cell adhesion and subsequent proliferation to provide high cell numbers required for the generation of new tissue. Interactions of cells with serum proteins that are adsorbed on biomaterial surfaces through cell membrane receptors have been shown to regulate cell adhesion and stem cell

differentiation (Chastain et al., 2006; Wang et al., 2013a). Chastain *et al.* showed that initial adhesion of MSCs to poly(lactide-co-glycolide) (PLGA) surfaces via collagen type I promotes osteogenesis while adhesion to poly(caprolactone) (PCL) via vitronectin does not (Chastain et al., 2006). Extracellular matrix (ECM) interactions with cells are not only of biochemical nature, biophysical cues such as topography, matrix stiffness, and dynamic forces also play a role in stem cell fate. The size of titanium oxide nanotubes on which cells were grown influenced MSC differentiation (Oh et al., 2009). Small-diameter (~30 nm) nanotubes promoted stem cell maintenance while larger (~ 70 to 100 nm) nanotubes induced osteogenic differentiation even without any additional osteogenic-promoting factors. Cells change their shape as they differentiate and lineage commitment of stem cells is dependent upon cell morphology. In a study where human MSCs were cultured on adhesive substrates that either promoted flat or round morphologies, osteogenesis was enhanced in cells that adhered and spread while adipogenesis was augmented in cells that did not spread (McBeath et al., 2004).

Another way to influence stem cell differentiation is by creating scaffolds that have similar mechanical properties than the tissue to be replaced. When the elastic moduli of collagen I coated polyacrylamide gels was adjusted to resemble brain, muscle, and bone tissues, MSCs differentiated towards the respective lineage (Engler et al., 2006). Similar results have been obtained when MSCs were encapsulated in three-dimensional RGD-modified alginate hydrogels of varying elastic moduli (Huebsch et al., 2010). Adipogenesis was predominant in softer gels (2.5-5 kPa), whereas elastic moduli of 11-30 kPa stimulated bone formation. Stem cells reside in a dynamic milieu in the human body where biochemical cues are presented in a spatially and temporally controlled manner. The generation of growth factor and oxygen

gradients to investigate optimal conditions for stem cell function can be achieved with microfluidic devices (Voog and Jones, 2010).

The *in vivo* stem cell niche, the bone marrow, is hypoxic in nature. Studies demonstrated that hypoxic environments (2% versus 20% oxygen) promote the proliferative capacity and also the plasticity of MSCs (Grayson et al., 2006). Biomaterials and scaffolds that possess biochemical and biophysical cues similar to the endogenous location of MSCs are required to successfully recreate stem cell niches *ex vivo*.

1.3.2 Biomaterials

Tissue engineers can choose from a big pool of materials consisting of natural polymers, synthetic polymers, combinations of both as well as inorganic materials such as ceramics and metals. Natural-derived polymer scaffolds are based on polysaccharides (alginate, chitin/ chitosan, hyaluronic acid derivatives) or proteins (collagen, fibrin gels, silk) (Chan and Leong, 2008). Most natural materials provide specific molecular recognition sites stimulating cell adhesion and function thus creating a biomimetic environment.

Collagen, a key protein in the ECM of many connective tissues, communicates with cells through integrin binding-mediated signaling. Its sequences are highly conserved across species boundaries explaining the low immunogenicity of collagens from xenogenic sources (e.g., bovine collagen) (Stover and Verrelli, 2011). Although natural materials have innate biological advantages, the possibility to obtain greater control over material properties and processing parameters led to the exploration of synthetic polymeric systems.

Most of the synthetic polymers are biologically inert. On the other hand, they are less prone to inflammatory host responses, possess well adjustable mechanical properties, and degradation rates. The most widely applied synthetic materials in stem cell engineering are

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polyacrylamid, polyacrylate, polyether, and polyester polymers (Marklein and Burdick, 2010).

Poly(ethylene glycol) diacrylate (PEGDA) is widely recognized as a biocompatible, non-immunogenic polymer. PEGDA surfaces resist protein adsorption and subsequently cell adhesion, thus providing a "blank environment" that can be modified with the incorporation of attachment peptides such as the Arg-Gly-Asp (RGD) peptide (Keskar et al., 2009d). The recreation of the natural ECM can also be realized by polymerizing poly(ethylene glycol) (PEG)-based hydrogels with natural biomaterials, such as collagen and hyaluronan (Park et al., 2003). PEG-based hydrogels have been explored for many TE applications including bone regeneration (Betz et al., 2010a), cartilage repair (Sharma et al., 2013) and for vascular tissue formation (Turturro et al., 2013).

Hydrogels are three-dimensional networks of hydrophilic polymer chains that are crosslinked either through physical, ionic or covalent interactions (Elisseeff, 2008). Because of the hydrophilic nature of polymer chains, hydrogels absorb water and swell in the presence of abundant water. Due to its structural similarities to ECM, hydrogels are particulary attractive for cell encapsulation (Tibbitt and Anseth, 2009). Material properties can be easily tailored based on changes in chemical structure, molecular weights, and crosslinking approach. PEGDA hydrogels are not degradable under physiologic conditions. To obtain hydrogels with physiologically relevant degradation profiles, PEG has been functionalized with degradable ester linkages typically lactide or glycolide segments or protease-sensitive peptides (Qiao et al., 2006; Turturro et al., 2013).

The last approach has been pioneered by Hubbell's group by integrating matrix metalloproteinase sensitive linkages into hydrogels via Michael addition (Patterson and

Hubbell, 2010). Cysteine-functionalized peptides react with acrylate, maleimide and vinyl sulfone groups of the respective monomer. PEG materials are inert and can serve as a basis for the presentation of organic molecules, peptides and proteins. For instance, MSCs could be differentiated towards osteogenic and adipogenic lineages in PEG hydrogels containing tethered small functional molecules (Benoit et al., 2008). Interestingly, phosphate groups containing hydrogels induced osteogenic differentiation and hydrophobic t-butyl group containing hydrogels upregulated adipogenic markers. Microfabrication techniques such as micromolding (Yeh et al., 2006), microfluidics (Mahadik et al., 2013), and bioprinting (Muller et al., 2013) have been applied to fabricate hydrogels with desired microarchitectures. However, PEGDA hydrogels are not restricted to microstructures and hydrogels with macroporous architectures have been created with porogen leaching (Chiu et al., 2013) or gas foaming techniques (Keskar et al., 2009d; Kollmer et al., 2012).

1.3.3   Superporous hydrogels as scaffold of choice

In the following thesis we evaluate if superporous PEGDA hydrogels (SPHs) promote human mesenchymal stem cell (hMSC) maintenance and controlled differentiation upon induction. The key attributes of the superporous hydrogel (SPH) as the name indicates are its accessible and interconnected pores ranging from 100 to 600 µm (Chen et al., 1999; Gemeinhart et al., 2001). The interconnected pores allow for fast incorporation of fluids by capillary force and, thus, enable a rapid and homogeneous cell uptake (Keskar et al., 2009d). The pores further provide extra space for tissue infiltration and vascular ingrowth, and enhance oxygen and nutrient transport (Guldberg et al., 2008). Without interconnected pores or innate degradability, cell penetration, communication and proliferation within scaffolds is inhibited (Chirila et al., 1993). Studies from our group and others showed that scaffold

architecture may be more important than chemical properties for cell survival (Kollmer et al., 2012), stem cell differentiation (Betz et al., 2010a) and vascularization (Brauker et al., 1995). We demonstrated that PEGDA-based SPHs, even in the absence of additional cell adhesion motifs, provide anchorage to MSCs, support their survival and osteogenic differentiation (Keskar et al., 2009d). Scaffolds with interconnected pores greater than 250 μm have been shown to promote angiogenesis (Druecke et al., 2004). Bai *et al.* observed no differences in the revascularization potential of scaffolds with pore sizes above 400 μm but the size of the pore interconnections determined the size and number of ingrowing blood vessels (Bai et al., 2010). Upon four weeks of transplantation into mice, neovascularization of acellular PEGDA SPHs was markedly enhanced when compared to the non-porous controls (Keskar et al., 2009b). In this thesis we will further investigate the revascularization potential of SPHs at earlier timepoints and employ stem cell-based strategies with the intend to enhance the *in vivo* angiogenic potential of SPHs. Another unique property of SPHs is their ability to maintain original shape during and after swelling. Therefore, SPHs have been investigated as gastric retentive devices in drug delivery applications targeted to the intestine (Chen et al., 2000). To recapitulate, we decided to investigate SPHs for stem cell-based TE applications due to their ability to support MSC attachment, viability and differentiation in addition to their capacity to promote the ingrowth of vascular structures. The latter is a prerequisite for cell survival after transplantation of the TE construct.

1.4 Vascularization in tissue engineering (TE)

1.4.1 The need for vascularization in TE

Engineered tissues are an alternative to autologous tissue transplantation. However, the success is limited due to poor survival of cells within scaffolds after transplantation. For

survival, metabolically active cells need to be within 150-200 μm distance from their nutrient source (Folkman and Hochberg, 1973). When delivery of nutrients is dependent upon diffusion, successful TE applications are currently limited to thinner tissues (< 2 mm) (Griffith et al., 2005). Larger tissues require vascular structures that provide oxygen and nutrients, as well as remove carbone dioxide and waste products.

Scaffold vascularization can be achieved by two main approaches: (1) stimulation of vessel ingrowth into avascular scaffolds with angiogenic factors or preseeded cells (neovascularization) (Lee et al., 2000), and (2) creation of vascular networks within the polymer scaffolds before implantation (prevascularization) (Chen et al., 2009b; Tremblay et al., 2005).

The first approach relies solely on the ingrowth of new vessels from the graft recipient. However, the formation of new blood vessels is a relatively slow process since endothelial cells migrate not faster than ~ 5 μm/h (Orr et al., 2003). Thus, cell death often occurs in the center of non-prevascularized TE constructs during the initial days after transplantation (Zhang et al., 2001). Alternatively, the prevascularization approach does not require fast host vessel infiltration since the scaffold contains preformed vascular structures that are thought to connect or anastomose with the host vessels after implantation. This approach can considerably accelerate vascularization within TE constructs and enhance cell survival if, both, the engineered vessels and the host vessels possess angiogenic sprouting activity and ultimately anastomose (Laschke and Menger, 2012).

1.4.2 The vascular system

1.4.2.1 General anatomy of blood vessels

Blood vessels are tubular structures that are composed of three concentric layers: tunica intima, tunica media and tunica adventitia (Rhodin, 2011). The innermost layer, the tunica intima, consists of a confluent endothelial cell (EC) monolayer lining the lumen and a thin layer of basal lamina. Large elastic arteries such as the human aorta additionally contain a subendothelial layer composed of collagen, elastic fibrils, smooth muscle cells (SMCs), and eventually some fibroblasts. The layer of thrombo-resistant ECs is called endothelium. It controls blood flow, vessel tone and vascular permeability (Bachetti and Morbidelli, 2000).

The tunica media is made up of SMCs (the only cell type in the media), collagenous and elastic fibrils. The SMCs also regulate blood flow by changing vessel diameter in vasodilation and vasoconstriction processes and the elastic fibers influence the visco-elastic behavior of the vessel.

The outermost layer of the vascular wall, the tunica adventitia, consists of dense fibroelastic tissue that contains adventitial fibroblasts, a collagen matrix, elastic fibers, and glycoproteins. The high collagen content gives most of the mechanical strength to the vessel. Small nutrient vessels such as arterioles, venules, blood capillaries, and lymphatic vessels, collectively referred to as vasa vasorum are also present in this layer. The adventitia also harbors nerves.

Generally, vascular structures can be divided into macrovessels (arteries and veins), microvessels (arterioles and venules), and capillaries. Arterioles vary in diameter from 10 to 300 μm and venules range from 10 to 50 μm. Capillaries, the smallest components of the vascular system, with an inner diameter of 4 to 10 μm have only an intimal layer. Whereas

macrovessels are separate anatomical entities, capillaries are structurally and functionally integrated within tissues and provide nourishment to regions that are not infiltrated by larger vessels (Ko et al., 2007).

1.4.2.2 The process of vascularization

Blood vessels are formed either through vasculogenesis, angiogenesis and arteriogenesis. During early embryonic development vessels need to be created *de novo* in previously avascular tissue during a process called vasculogenesis (Carmeliet, 2000). Undifferentiated endothelial progenitor cells (EPCs) mature to ECs to create an initial vascular network. The primary vascular network consists of several nascent endothelial tubes that are composed of polarized ECs (Ko et al., 2007).

During angiogenesis, capillaries enlarge, sprout or bridge to form more complex structures. The angiogenic process requires an intimate interplay between cells, growth factors, and extracellular matrix (ECM) to initiate neovascularization. ECs usually organize into vascular networks by tip-cell extension in response to a growth factor gradient that originates in ischemic areas and causes EC activation (Saik et al., 2012). The activated tip cell releases matrix metalloproteinases (MMPs) that cause degradation of basement membrane. ECs proliferate and migrate into the degraded areas to form vacuoles and fuse to tubular structures. Immature tubes differentiate into capillaries after connecting with pericytes (PCs) whereas larger vessels such as arteries and veins are composed of SMCs. Pericytes produce basement membrane proteins and, like SMCs, also have contractile elements to regulate blood flow (Bergers and Song, 2005; Orlidge and D'Amore, 1987). Besides contributing to the stability and maturation of newly formed vessels, PCs also turn EC proliferation down to their quiescent state.

Arteriogenesis refers to changes in lumen diameter or wall thickness in preexisting small arterioles. Vascular remodelling occurs in response to mechanical or chemical stimuli (Cassell et al., 2002).

1.4.3 Prevascularization of tissue engineering scaffolds

During this approach scaffolds are pre-seeded with capillary-forming cells such as endothelial cells (ECs) or cocultures of ECs and other cell types such as fibroblasts, osteoblasts and mesenchymal stem cells. Prior to implantation, the constructs are matured *in vitro* until vascular structures are formed. Interconnected 3D capillary systems, lumens and contiguous vessel walls have been observed in various biomaterials (Black et al., 1998; Duffy et al., 2011). Regardless of the applied biomaterial, the stimulation of microvascular networks that allow perfusion is critical for the survival of newly formed tissue. Ideal scaffolds for vascularization approaches should enable the formation of capillary-like structures from implanted cells and facilitate rapid ingrowth of vascularized tissue from the host.

Vessel-like structures in prevascularized scaffolds can be created either by angiogenesis or vasculogenesis and connect with host vessels in a process called wrapping-and-tapping (WAT) anastomosis (Cheng et al., 2011). Engrafted ECs wrap around the ingrowing host vessels, and cause degradation of the host endothelium to redirect blood flow to the engineered vascular network. High expression levels of matrix metalloproteinase-(MMP)-14 and MMP-9 have been shown to accompany this process. This finding can be considered in the design of new TE scaffolds. Proper manipulation of local MMP levels might lead to faster perfusion of those constructs.

In general, it is hypothesized that prevascularization of TE constructs results in a better connection with the host vasculature and a faster vascularization rate after implantation. In a fibrin gel that was prevascularized with HUVECs and fibroblasts (cell ratio 5:1) for 1 week, vessels from the host could be detected 5 days after implantation whereas the same process took 14 days in the non-prevascularized gel (Chen et al., 2009b). When HUVECs were coseeded with a high density of fibroblasts (cell ratio 1:5) in the same TE construct anastomosis with the host vasculature could be accelerated by 2 or 3 days (Chen et al., 2010). *In vivo* prevascularization represents another strategy to improve cell survival in TE constructs. Prevascularization of a cardiac cell-seeded alginate scaffold on the omentum, a blood vessel enriched membrane, for 7 days resulted in the generation of a functional blood vessel network (Dvir et al., 2009). The engineered cardiac patches were fully integrated and electrically coupled with the rat myocardium 28 days after transplantation onto the infarct side suggesting a benefit of the prevascularization approach.

1.5 Outline, rationale and hypotheses

We have previously established a unique method for superporous hydrogel (SPH) formation in our group (Keskar et al., 2009d). Human mesenchymal stem cells (hMSCs) that were seeded within the porous network survived over a month and mineralized tissue within PEGDA-based SPHs (Keskar et al., 2009d). Hydrophilic PEGDA hydrogels are generally thought to be unsuitable for anchorage-dependent cells to adhere due to their nonadhesive nature. Interestingly, our previous findings revealed that unmodified PEGDA SPHs allow anchorage of bone marrow derived hMSCs and support their long-term survival. Therefore, we hypothesized that the physicochemical characteristics of the SPHs hold properties that could foster cellular responses. The objective of the first part of this thesis was to investigate

whether hMSCs are able to modify the cell-repellant PEGDA environment to a milieu conducive to stem cell survival and function (Chapter 2). We conducted immunofluorescence staining and real-time PCR to examine if hMSCs are able to synthesize their own extracellular matrix (ECM) proteins within the SPHs. Since the ECM not only supports hMSCs vitality but also promotes their stem state, we investigated if the hMSCs stay undifferentiated after being cultured within the SPH for an extended period and if they are capable to undergo controlled differentiation after osteogenic, chondrogenic and adipogenic induction.

Many in the field of tissue engineering utilize a small subset of gene markers to indicate stem cell differentiation towards a certain lineage despite the possibility for expression of these markers in multiple lineages. Due to the heterogeneous nature of hMSCs and the known plasticity between adipogenic and osteogenic lineages (Schilling et al., 2007), we hypothesized that gene markers overlap between hMSC-derived adipocytes and osteoblasts. Thus, we evaluated adipocyte- and osteoblast-associated genes for their suitability to indicate differentiation towards the respective lineage (Chapter 3).

Another major challenge of engineered tissue lies in the vascularization and in identifying appropriate cell sources that support the endothelialization of vascular implants. We previously observed the ingrowth of vascular tissue in acellular SPHs after 4 weeks of implantation into mice (Keskar et al., 2009b). In this thesis, we further investigated the role of mesenchymal stem cell-derived endothelial cells on vascularizing SPHs after implantation onto the chick chorioallantoic membrane (CAM) for 7 days (Chapter 4). Preseeding of scaffolds with endothelial-like MSCs prior to implantation has been shown to effectively promote neovascularization of grafts (Janeczek Portalska et al., 2012; Sahar et al., 2012).

Thus, <u>we hypothesized</u> that endothelial-like hMSCs can be used to improve SPH vascularization. In the last chapter conclusions are drawn and future studies are suggested (Chapter 5).

Table 1.1 Protocols used for *in vitro* endothelial differentiation of MSCs.

MSC origin	Media	Growth factors	Cultivation time [days]	Marker expression after differentiation	Ref.
Bone marrow	DMEM, 2% FCS	50 ng/ml VEGF	7	Pos.: FLT-1, KDR, VE-cadherin, VCAM-1, vWF Neg.: CD31, CD34	(Oswald et al., 2004)
Bone marrow	EGM-2	-	21	Slightly pos.: CD31, CD34, VE-cadherin, KDR	(Liu et al., 2007)
Bone marrow	IMDM, 5% FBS	50 ng/ml VEGF	14	FLT-1, KDR, vWF	(Zhang et al., 2008)
Bone marrow	EGM-2 + shear force	-	10	CD31, KDR	(Janeczek Portalska et al., 2012)
Adipose tissue	Medium 199, 3% FBS	50 ng/ml VEGF, 10 ng/ml b-FGF	2	CD31, CD34, eNOS, VE-cadherin	(Cao et al., 2005)
Adipose tissue	DMEM, 2% FCS	50 ng/ml VEGF-C	8	IF: CD31 WB: vWF	(Sahar et al., 2012)
Umbilical cord	DMEM-LG, 5% FBS	100 ng/ml VEGF, 50 ng/ml EGF, 1 µg/ml hydrocortisone	up to 12	KDR, VE-cadherin, vWF	(Chen et al., 2009a)
Placenta	EGM-2	50 ng/ml VEGF-A	14-21	CD31, CD34, FLT-1, KDR, VE-cadherin, vWF	(Lee et al., 2009)
Amniotic membrane	DMEM, 2% FBS	50 ng/ml VEGF-A	7	CD34, FLT-1, ICAM-1, KDR, vWF	(Alviano et al., 2007)

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2 Stem Cell-Derived Extracellular Matrix Enables Survival and Multi-Lineage Differentiation within Superporous Hydrogels

The contents of this chapter are based entirely on the following article: Köllmer M, Keskar V, Hauk TG, Collins JM, Russell B, Gemeinhart RA. Stem Cell-derived Extracellular Matrix Enables Survival and Multi-Lineage Differentiation within Superporous Hydrogels. Biomacromolecules,13(4): 963-973, 2012. This article is reprinted with the permission of the publisher and is available using DOI: 10.1021/bm300332w. Copyright 2012. American Chemical Society.

2.1 Introduction

Human mesenchymal stem cells (hMSCs) derived from adult tissues have shown increasing potential in regenerative medicine due to their multilineage differentiation capability and their immunomodulatory effects (Caplan, 2009; Djouad et al., 2009; Kuhn and Tuan, 2010). In the human body MSCs ensure homeostasis by supporting the repair and rejuvenation of degenerated tissues and organs. To induce repair, hMSCs undergo differentiation into a variety of mature cell types including osteoblasts, chondrocytes and adipocytes and secrete bioactive factors as "trophic mediators" that alter the milieu of dysfunctional tissues (Caplan and Dennis, 2006b; Pittenger et al., 1999). Current tissue engineering approaches typically involve the development of stem cell-carrying extracellular matrix (ECM)-mimicking scaffolds that mature *in vitro* before implantation into the patient. To control for lineage specific differentiation, ideal scaffold materials should be able to maintain stem cells in their undifferentiated phenotype and promote differentiation only after induction.

Our group has demonstrated that poly(ethylene glycol) diacrylate (PEGDA) superporous hydrogels (SPHs) promote long-term survival and mineralization of hMSCs *in vitro* (Keskar et al., 2009c). Host cell infiltration and neovascularization of hMSC-seeded SPHs was also observed *in vivo* (Keskar et al., 2009a). SPHs were fabricated utilizing a gas foaming

technique, wherein the foaming and gelation processes occur simultaneously to create an interconnected macroporous network with pores ranging from 100 to 600 μm (Keskar et al., 2009c). The interconnected pores allow for fast absorption of fluids by capillary force and, thus, enable a rapid cell uptake. However, hydrophilic PEGDA hydrogels are considered resistant to cell binding due to poor protein adsorption to the surface (Nuttelman et al., 2005). The viability of cells encapsulated within unmodified non-porous PEGDA hydrogels drops to 15% within one week of culture (Nuttelman et al., 2005). We have recently reported that cells can be incorporated into the hydrogel matrix of superporous hydrogels despite the harsh foaming and polymerization conditions (Desai et al., 2012). These conditions do limit which cell types can be incorporated into the matrix of the SPHs, but leave open the possibility for dual cell type incorporation. Based upon this, there is potential to form these hydrogels *in situ* with cell encapsulation within the matrix followed by loading of cells within the pores.

Interestingly, our previous findings revealed that PEGDA SPHs, even in the absence of cell adhesive peptides, provide anchorage to hMSCs and support long-term survival suggesting that the porous environment *per se* imparts properties that could endorse cellular responses. Macroporous degradable PEG hydrogels have been shown to promote hMSC osteoblast differentiation by facilitating cell communication through autocrine and paracrine signaling via the interconnected pore network (Betz et al., 2010b). Importantly, PEGDA SPH synthesized with an anionic monomer, *i.e.* acrylic acid, eliminated cell adhesion without altering the macroscopic structure, indicating that the observed cell binding phenomenon is not entirely due to the hydrogel architecture (Kadakia et al., 2008).

Initial anchorage of cells to implant materials occurs *via* binding to proteins that are either immobilized on the substrate or adsorbed from the serum in culture. Cell-surface integrins

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subsequently bind to amino-acid sequences, *e.g.* Arg-Gly-Asp (RGD), in the adsorbed protein molecules (Frisch et al., 1996). Anchorage-dependent cells also deposit their own ECM molecules after seeding on matrices that do not possess any natural binding motifs to prevent anoikis, a form of apoptosis induced by the lack of sufficient cell-ECM contact (Chiarugi and Giannoni, 2008; Frisch and Francis, 1994). In 2D monolayer cultures, undifferentiated hMSCs secrete collagen I, collagen IV as well as adhesive proteins such as fibronectin and laminin (Chichester et al., 1993; Conget and Minguell, 1999; Prockop, 1997). The secreted ECM provides environmental cues for cell survival, proliferation and differentiation. *In vivo*, cell adhesion molecules play an important role in holding the stem cells within their specific niche and thereby allowing cell-cell and cell-ECM interactions. In the current study, we cultured hMSCs in basal medium for a period of 3 weeks within the PEGDA SPHs and monitored the expression of ECM molecules by immunofluorescence and real-time qPCR to reveal cell-scaffold interactions that may influence attachment.

Since the ECM not only promotes hMSCs vitality but also helps to maintain their stem state (Matsubara et al., 2004), we investigated if the hMSCs stay undifferentiated after being cultured within the SPH for an extended period of 3 weeks and if they are able to undergo controlled differentiation after osteogenic, chondrogenic and adipogenic induction. The maintenance of hMSCs in their undifferentiated state allows for site-specific cell responses, and thus could be advantageous in many tissue engineering applications where optimal tissue integration is required. To this end, the objective of the present study was to investigate whether hMSCs are able to alter the cell-repellant PEGDA environment to a milieu conducive to cell growth and multi-lineage differentiation by secreting their own adhesive proteins within the SPH scaffolds.

2.2 Materials and Methods

2.2.1 Materials

PEGDA (3,400 MW) was obtained from Laysan Bio, Inc. (Arab, AL). Pluronic® F127 was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). N,N,N',N'-tetramethylethylenediamine (TEMED, 99%, Acros Organics), ammonium persulfate (APS, 98+%, Acros Organics), citric acid anhydrous, sodium bicarbonate and paraformaldehyde (96%, Acros Organics) were purchased from Fisher Scientific (Fair Lawn, NJ). Chemicals were used as received without any further purification. Dulbecco's Modified Eagle's Medium, trypsin-EDTA, penicillin and streptomycin were from Mediatech, Inc. (Cellgro®, Manassas, VA). Foundation™ fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). Primers were ordered from Integrated DNA Technologies (Skokie, IL) (Table 2.1).

2.2.2 Fabrication of superporous hydrogels

Scaffolds with pore sizes ranging from 100 to 600 μm were prepared by a gas foaming method as previously reported (Keskar et al., 2009c). Briefly, aqueous PEGDA solution (15 (w/v) %), foam stabilizer (Pluronic® F-127; 0.6 (w/v) %), and the initiator pair N,N,N',N'-tetramethylethylenediamine (TEMED; 0.9 (v/v) %) and ammonium persulfate (APS; 0.7 (w/v) %) were added sequentially to a glass vial to a final volume of 1 mL. Saturated citric acid solution (2 (w/v) %) was used to adjust the pH to 3.70. The solution was heated at 37-40°C for 2 minutes. Sodium bicarbonate (200 mg) was stirred into the precursor solution evolving CO_2 by reacting with the citric acid. Polymerization occurred simultaneously as the pH increased to a pH of approximately 7. Polymerization was allowed to proceed for 30 min. SPHs were then removed from the vial and washed three times in double-deionized water to remove traces of unpolymerized monomers and salt before sterilizing the gels in 80% ethanol

overnight, followed by dehydration in absolute ethanol for 4 hours. SPHs were dried in a food dehydrator at a temperature of 55°C for approximately 2 hours. Dried hydrogels were cut to give scaffolds with a diameter of 5 mm and a thickness of 3 mm.

2.2.3 Human mesenchymal stem cell isolation, seeding and cultivation

Human bone marrow aspirates were obtained from AllCells, LLC (Emeryville, CA) and isolated by density gradient centrifugation utilizing Ficoll-Paque™ PLUS solution and RosetteSep® Human Mesenchymal Stem Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's protocol. For each experiment, hMSCs from 3 different donors, up to passage 4, were used. Cells were harvested using 0.25% trypsin with 1.0 M EDTA, centrifuged and expanded in basal medium which consists of high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 unit/mL streptomycin. DMEM without FBS was used for serum-free culture. For cell seeding, dehydrated SPH cylinders were placed into 48 well plates. Cell suspension (50 μL) containing 2.5×10^5 cells (unless stated otherwise) was added drop-wise to each SPH. The hydrogels were placed in the incubator for 30 min to allow equilibrium swelling and initial cell attachment. Thereafter, 1.0 mL of basal medium was added to each of the wells, and the plates were incubated at 37°C in 5% CO_2. Medium was changed every third day. Cell-seeded constructs were cultured for 3 weeks in basal medium before differentiation medium was added for additional 4 weeks of culture. Adipogenic differentiation was initiated by culturing the cell-seeded constructs in adipogenic induction medium (AI) for 3 days, following treatment with adipogenic maintenance medium (AM) for the next 3 days. The alternation between AI and AM treatment was continued for the 4 weeks of culture. Chondrogenic differentiation was induced by serum-free

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chondrogenic induction medium (Lonza, Walkersville, MD) containing dexamethasone and TGF-β3. Osteogenic differentiation medium (Lonza, Walkersville, MD) consisted of basal medium with 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic acid 2-phosphate. Controls were maintained in basal medium for the entire culture period of 7 weeks.

2.2.4 Cell viability

Cell viability was analyzed immediately after seeding (day 0), and 1, 7, 14, 21 and 28 days after seeding hMSCs at a density of 5×10^3 cells/cm^2 on 48 well plates (3750 cells/well) and within PEGDA SPHs (2.5×10^5 cells/SPH). To examine the proliferative activity in serum-free culture, serum-containing medium was switched to serum-free medium 24 hours after seeding. The MTS assay measures cell viability as a function of mitochondrial activity. MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] is reduced into a colored formazan product by metabolically active cells. In brief, 200 μL of 1X DPBS and 40 μL of Cell-Titer 96® Aqueous One Solution reagent (Promega, Madison, WI) were added to each well. The plates were covered and incubated for 3 h at 37 ºC. Supernatant (100 μL) was transferred to a new plate and absorbance was measured at 492 nm (Labsystems Multiskan Plate Reader). The controls for background absorbance were unseeded SPHs.

For live-dead staining, the wells were rinsed with 1X DPBS to remove serum and medium components. A solution comprising 2.5 μL calcein acetoxymethyl ester (4 mM) and 2.5 μL ethidium homodimer (2 mM) in 5 mL of 1X DPBS was added to the SPHs and allowed to incubate for 25 min at 37°C. SPHs were rinsed twice with 1X DPBS and fluorescent images were taken with an Olympus IX70 inverted microscope.

2.2.5 Quantitative real-time PCR

At specific timepoints, *i.e.* 24 hours, 3 and 7 weeks after seeding hMSCs at a density of 5 x 10^3 cells/m^2 on 6 well plates (5 x 10^4 cells/well) and within PEGDA SPHs (7.5 x 10^5 cells/SPH), total RNA was extracted using the TRIzol$^®$ reagent (Invitrogen) in combination with the PureLink^{TM} RNA Mini Kit (Invitrogen) according to manufacturer's instructions. RNA (180 ng) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The PCR reactions were performed on an Applied Biosystems StepOnePlus^{TM} PCR machine using 5 µL SYBR$^®$ Green PCR Master Mix (Applied Biosystems), 2 µL sequence specific primers (0.5 mM, Table 2.1) and 3 µL cDNA under the following conditions: 95°C for 10 min followed by 40 cycles of 15 s of denaturation at 95ºC and 60 s of annealing and elongation at 60ºC. A melting curve analysis was performed after each run to confirm product specificity. The delta-delta-Ct method (Schmittgen and Livak, 2008) was employed to determine the relative gene expression level of the gene of interest normalized to the expression of the endogenous control ribosomal protein L13 α (RPL13α) (Curtis et al., 2010a).

2.2.6 Flow cytometry

HMSCs were cultured on 6 well plates (5 x 10^4 cells/well) or within SPHs (7.5 x 10^5 cells/SPH) for 21 days in basal medium. To remove hMSCs from the scaffolds, the cell-seeded constructs were incubated with trypsin-EDTA (0.25% trypsin, 2.21 mM EDTA) for 5 min under slight shaking. After centrifugation, cells were incubated on ice for 30 min in 100 µL of FACS buffer (1X DPBS + 5% FBS + 0.05% 3M sodium azide) with anti-human CD105-FITC, CD90-FITC, CD73-APC or CD44-PE. Cells were washed twice with FACS buffer and finally diluted into 100 µL of FACS buffer. Fluorochrome- and isotype-matched

antibodies were used as controls. All antibodies were obtained from Biolegend (San Diego, CA) and used at manufacturer's recommended concentrations. Analysis was performed by collecting 15, 000 events on a Beckman Coulter Cyan ADP.

2.2.7 Immunofluorescence

Immunostaining was carried out to detect and visualize the expression of collagen type I and IV, laminin, and fibronectin. Briefly, the samples were rinsed with 1X PBS to remove medium components. Following fixation with 3.7% paraformaldehyde for 15 min, the samples were treated with blocking solution (1% BSA in 1X PBS). After 30 min incubation on a shaker, respective primary antibodies (5 µg/ml anti-collagen type I; 4 µg/ml anti-collagen type IV; 2 µg/ml anti-laminin, 2 µg/ml anti-fibronectin) were added to the blocking solution and samples were further incubated for 3 hours at room temperature (RT). Mouse monoclonal IgG antibodies (Santa Cruz Biotech, Santa Cruz, CA) were used for all ECM molecules. Samples were washed with 1X PBS and incubated with a FITC-labeled goat anti-mouse secondary antibody (5 µg/ml; Molecular Probes, Carlsbad, CA) for 20 min at RT. The nucleus in the 2D samples was counterstained with H33258 (0.5 mg/ml; Molecular Probes, Carlsbad, CA) for 15 min at RT. Appropriate mouse control IgG antibodies were used at dilutions corresponding to the primary antibody (data not shown). Images were taken with an Olympus IX70 inverted microscope and processed using IPLab^{TM} software.

2.2.8 Histochemical confirmation of differentiation

Sudan III staining was applied to stain lipid vacuoles to evaluate adipogenic differentiation. The cell-seeded constructs were rinsed with 1X PBS (pH 7.4) and incubated with Sudan III solution (0.3% w/v of Sudan III in 70% ethanol) for 3 minutes. After several washes with double deionized water (DDIW), harris hematoxylin solution was added and incubated for 1

minute. The wells were rinsed with DDIW until the water ran clear. Images were taken under bright field with an Olympus IX70 inverted microscope.

Safranin O staining was used for the detection of glycosaminoglycans to evaluate chondrogenic differentiation. In brief, cell-seeded constructs were washed with 1X PBS (pH 7.4) and fixed with 3.7% paraformaldehyde solution at room temperature. After rinsing with 1X PBS, 0.01 % aqueous fast green was added to the sample and incubated for 3 minutes. The sample was washed twice with 1% acetic acid and incubated with aqueous Safranin O solution (0.1 %) for 5 minutes. The wells were rinsed with DDIW till the water ran clear. Images were taken under bright field with an Olympus IX70 inverted microscope.

To evaluate osteogenic differentiation calcium was determined by complexation of calcium by the phenolsulphonephtalein dye (Woo and Cannon, 1991). Briefly, the lyophilized hydrogels were homogenized with 1.0 mL of 0.5 N HCL and mixed overnight at 4ºC. The supernatant was used for calcium assays, as per manufacturer's protocol (QuantiChrom^{TM} Calcium assay kit, BioAssay Systems).

2.2.9 Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test (Origin 8.1) if the means were significantly different at a p-value of 0.05. All data are presented as mean plus or minus standard deviations.

2.3 Results

2.3.1 MSC viability and proliferation

Viable cells with an elongated spindle-shaped, fibroblast-like morphology could be observed within the SPHs during the entire test period of 7 weeks (**Figure 2.1 a,b**). Serum is

necessary for hMSC expansion under 2D conditions on tissue culture plastic, but hMSCs cultured within SPHs proliferate similarly in the presence or absence of serum in basal medium, as assessed by the MTS assay (**Figure 2.1c**) and mRNA expression levels of proliferating cell nuclear antigen (PCNA) (**Figure 2.1d**). The PCNA mRNA levels of hMSCs cultured for 3 weeks within SPHs remained constant under both, serum-supplemented and serum-free conditions, while PCNA mRNA levels from hMSCs expanded under 2D conditions was initially high and decreased significantly from day 1 to day 21 to a level similar to that of SPH samples. The differences between the MTS assay results and the gene expression patterns of PCNA may arise form the fact that cells grow to a confluent state on the tissue culture plate and stop proliferating at later time points which results in decreased mRNA expression of PCNA. Additionally, it appears that there is less signaling to proliferate within the SPHs leading to a stable number of cells within the structures.

2.3.2 Influence of serum on MSC attachment within PEGDA SPHs

In order to delineate the role of serum proteins in aiding hMSC attachment and survival within PEGDA SPHs, hMSCs were cultured in complete medium and serum-free medium. HMSCs seeded in SPHs and cultured in complete DMEM were viable and displayed an extended morphology (**Figure 2.2a-b**). Within 24 hours of seeding, the cells were extending between clusters suggesting cell-cell interactions. When the cell-seeded constructs were transferred to serum free culture for the next 24 hours, the hMSCs retained their viability (**Figure 2.2c**). In contrast, when the cell-seeded constructs were directly cultured in serum-free conditions, no viable cells could be found within the SPHs at the end of 24 hours (**Figure 2.2d**). These results indicate that the presence of serum proteins is required for initial anchorage of hMSCs within the SPHs but not for the hMSC survival after 24 hours.

2.3.3 Matrix production within superporous hydrogels

The deposition of ECM molecules by hMSCs that were cultured either within PEGDA SPHs or on 2D tissue culture plastic was examined by immunofluorescence staining (**Figure 2.3**) and real-time qPCR (**Figure 2.4**). At the end of 3 weeks, hMSCs were embedded in a dense matrix of collagen type I, collagen type IV, laminin, and fibronectin in both culture systems (**Figure 2.3**). In 2D culture, the cell-deposited ECM was localized around the nuclei and displayed a fibrous pattern. In the PEGDA SPHs, hMSC-secreted ECM molecules were mainly localized around the porous network with similar fibrillar structures (**Figure 2.3**). Since assessment of the protein expression with immunofluorescence staining in the SPH is not quantitative, we conducted real-time qPCR to quantify gene expression of ECM molecules.

Collagen type I, collagen type IV and fibronectin mRNA expression levels of hMSCs seeded within PEGDA SPHs were similar to the expression levels of hMSCs expanded in 2D monolayer for 21 days in serum-containing basal medium. At the earliest time point, laminin expression was significantly upregulated ($p = 0.03$) for hMSCs cultured in SPHs compared to on TCP in serum-supplemented media and slightly upregulated, but not statistically different ($p = 0.08$), in serum-supplemented media at day 21.

Interestingly, the absence of serum did not have any significant effects on the gene expression of ECM molecules within the SPH culture system. As well, the mRNA levels of ECM molecules remained relatively constant over the 21 days of culture within the PEGDA SPHs. Whereas the mRNA levels of collagen type I, collagen type IV and fibronectin decreased from day 1 to day 21 in 2D monolayer in the presence of serum ($0.001 < p < 0.05$). The decrease in ECM molecule gene expression can be explained by the fact that hMSCs

grew confluent within 21 days of culture on the 2D plate and do not need to synthesize more ECM for cell attachment. Similar to SPHs, the hMSCs did not proliferate significantly under 2D serum-free conditions (**Figure 2.1c**) and the gene expression did not change significantly from day 1 to day 21.

2.3.4 Surface marker expression within superporous hydrogels

Flow cytometry was employed to assess the surface marker profiles of passage 4 hMSCs cultured for 21 days within SPHs or on 2D TCP (**Figure 2.5**). The mean percent expression of CD105 was below 80% whereas CD90, CD73 and CD44 expression levels were over 95% in both culture systems (**Table 2.2**). Since the mesenchymal surface marker expression was not significantly altered after the transfer to the 3D system, we conclude that PEGDA SPH culture supports the undifferentiated state of hMSCs and eventually preserves their tri-lineage differentiation potential. Flow cytometry of hMSCs from passage 2 revealed similar CD105 expression levels of 78.4% ± 1.2%. Comparable results for the CD105 expression (88.1% ± 7.4%) within unsorted bone marrow derived hMSCs have been reported (Kern et al., 2006).

2.3.5 Multipotency of MSCs within superporous hydrogels

To examine whether hMSCs are able to retain their multilineage differentiation potential after cultivation in the SPHs, we cultured the cell-seeded constructs (2.5×10^5 cells/SPH) for 3 weeks in basal medium, followed by treatment with adipogenic, chondrogenic and osteogenic differentiation medium for a period of 4 weeks. Controls were maintained in basal medium for the entire culture time of 7 weeks (**Figure 2.6a**). Lipid vacuoles that stained bright red with the lipophilic dye, Sudan III, could be observed within PEGDA SPHs upon exposure to adipogenic medium. No lipid vacuoles could be detected within the non-induced

controls (**Figure 2.6b**). The presence of proteoglycans was confirmed by Safranin O staining and could only be detected in the chondrogenic induction group (**Figure 2.6b**). Cell-seeded constructs cultured in osteogenic medium showed significantly elevated calcium levels compared to the non-induced control groups (**Figure 2.6c**).

The relative expression of fat-related genes, adiponectin (*ADIPOQ)* and leptin (*LEP)* (**Figure 2.6d-e**), cartilage-related genes, collagen type II (*COL2A1)* and aggrecan (*ACAN)* (**Figure 2.6f-g**), and bone-related genes, alkaline phosphatase (*ALPL)* and osteocalcin (*BGLAP)* (**Figure 6h-i**) was examined by real-time qPCR. The gene expression of adiponectin, leptin and collagen type II exhibited similar patterns: significant higher mRNA levels in the induction groups at week 7 compared to the control groups at day 1, week 3 and week 7. Aggrecan mRNA expression levels were significantly elevated after 3 and 7 weeks of SPH culture compared to day 1 but no significant differences between the control groups and the chondrogenic induction group was observed for week 7. The expression of bone markers, alkaline phosphatase and osteocalcin, showed a decreasing trend over the 7 weeks of SPH culture and no significant differences between the control groups and the osteogenic induction group was seen which contradicts the data observed for calcification (**Figure 2.6c**).

2.4 Discussion

The goal of this study was to investigate cell-matrix and cell-polymer interactions of human mesenchymal stem cells (hMSCs) cultured within PEGDA superporous hydrogels (SPHs). Since PEG surfaces are thought to be devoid of cell-matrix interactions, we hypothesized that the physicochemical characteristics of the SPH and its macroporous architecture affect cellular responses. Our results demonstrated that macroporous PEGDA hydrogels provide a microenvironment that supports stem cell-derived extracellular matrix

(ECM) development. The secreted ECM together with the hydrogel surrounding promoted stem cell maintenance and multi-lineage differentiation.

Over a period of 7 weeks viable cells were detected throughout the PEGDA SPHs (**Figure 2.1**). The majority of hMSCs exhibited a spindle-shaped, fibroblast-like morphology. For hMSCs, a thin stellate or spindle-shaped morphology is suggestive of an undifferentiated state (Sekiya et al., 2002). In contrast, when encapsulated within nonporous 3D PEGDA hydrogels that are not modified with RGD, hMSCs are forced into a rounded morphology with limited capability to spread, and consequently undergo apoptosis (Nuttelman et al., 2005; Pierschbacher and Ruoslahti, 1987). In addition, hMSCs do not survive on top of unmodified, nonporous PEGDA hydrogels, which could serve as a control for the porous architecture of the SPH with similar surface chemistry (Keskar et al., 2009c). Thus, it is proposed that the interconnected porous architecture of the PEGDA SPHs promotes long-term survival of hMSCs. Furthermore, there are also chemical and physical components to the ability to populate the scaffolds and create a niche as hMSCs grown in SPHs with similar mechanical properties and morphology were not able to survive for even 24 hours without incorporation of collagen (Kadakia et al., 2008). SPHs containing acrylic acid in their formulation were "repulsive" to cell attachment indicating significantly altered protein and cell interactions with the hydrogel.

It is suggested that 3D scaffold culture turns cell proliferation down (Duggal et al., 2009). Duggal *et al.* showed that hMSCs encapsulated within 2% RGD-alginate gels did not proliferate although they remained viable over the 21 days study period (Duggal et al., 2009). Gene expression of PCNA, encoding for proliferating cell nuclear antigen that is expressed in the nuclei of cells during DNA synthesis, remained constant in hMSCs cultured for 3 weeks

within PEGDA SPHs in the presence and absence of serum (**Figure 2.1c**). The slow proliferation rate of hMSCs in SPHs compared to their extensive growth under 2D conditions is actually more similar to their *in vivo* behavior where adult stem cells produce progeny at lower dividing capacity (Scadden, 2006).

Whereas serum is needed for hMSC expansion under 2D conditions on tissue culture plastic (TCP) (**Figure 2.1c**), hMSCs cultured within SPHs proliferate similarly in the presence or absence of serum in the basal medium (**Figure 2.1c**), which could have the additional benefit for human cells of avoiding the use of animal serum for culture. We hypothesized that the cells must be secreting their own ECM within the SPHs that may control cell adhesion, proliferation and differentiation.

Cell adhesion to polymer surfaces is mediated by proteins that have been immobilized onto the substrate, adsorbed from the surrounding medium or secreted by the cells themselves. Initial attachment of hMSCs to PEGDA SPHs is mediated by serum proteins (**Figure 2.2**) that have been adsorbed onto the scaffold surface. HMSCs cultured in serum containing medium are able to anchor to the SPHs and thus overcome initial pro-apoptotic signals that lead to *anoikis* of anchorage-dependent cells (Chiarugi and Giannoni, 2008; Frisch and Francis, 1994). However, this effect was not seen when the hMSCs were cultured on the nonporous hydrogels. Besides initial anchorage dependent upon serum proteins, integrin-mediated signaling via attachment to ECM is required to maintain cell viability (Frisch et al., 1996).

Undifferentiated hMSCs secrete various ECM proteins that are part of the specialized niche that controls stem cell behavior (Chen et al., 2007). Immunofluorescence staining revealed expression of collagen type I, collagen type IV, fibronectin and laminin within hMSC-seeded

PEGDA SPHs by the end of three weeks (**Figure 2.3**). Endothelial cells seeded onto RGD-modified PEGDA hydrogel surfaces were able to attach and secrete ECM even after the RGD recognition sequences had been hydrolyzed from the system (Elbert and Hubbell, 2001). The cell sheet did not detach from the hydrogels indicating that, after initial attachment due to the RGD ligands, the cells had secreted ECM that was interacting strongly with the PEGDA surface (Elbert and Hubbell, 2001). Rather than direct attachment to the entangled PEGDA chains, it is hypothesized that, the secreted fibronectin filaments and collagen fibrils diffuse within the polymer chains and form an inter-penetrating network with the PEGDA hydrogels, which have a mesh size on the order of 6 nm (King et al., 2002). Although the staining clearly showed ECM, it is unclear how this ECM interacts with the PEGDA hydrogels if at all.

To monitor ECM development, we examined gene expression of hMSCs cultured within PEGDA SPHs and on 2D surfaces. Collagen type I, collagen type IV and fibronectin mRNA expression levels of hMSCs seeded within PEGDA SPHs were similar to the expression levels of hMSCs expanded in 2D monolayers for 21 days in basal medium (**Figure 2.4a-c**). Laminin (*LAMA5*) expression was slightly upregulated in the SPH at day 1 compared to 2D TCP (p=0.03) but no significant difference was observed at day 21 (p=0.08) (**Figure 2.4d**). Interestingly, the absence of serum did not have any significant effects on the gene expression of ECM molecules within the SPH culture system. We believe that the cell-secreted ECM supports long-term survival of hMSCs within the PEGDA SPHs and aids in creating a niche environment conducive to stem cell maintenance and multi-lineage differentiation after induction with appropriate cues. The deviation in the ECM expression between 2D and SPH culture may arise from the fact that cells sense different architectural

and topographical cues. However, alterations in surface chemistry, polystyrene tissue culture plastic versus poly(ethylene glycol) diacrylate hydrogel substrata, might have also played a role. As stated earlier, we are unable to grow cells directly on 2D PEGDA hydrogels, so comparison is not possible. But, it is clear that there are differences in the amount of mRNA being produced for each of the proteins examined, with laminin in particular being elevated in the SPH cultured hMSCs.

Laminin, known for its role in embryogenesis, has been shown to support human embryonic stem cells (hESCs) proliferation and stem cell maintenance (Miyazaki et al., 2008). In adhesion assays with ECM protein-coated tissue culture plates, more than 60% of the hMSCs bind to fibronectin, collagen type I, and collagen type IV, and about 30% bind to laminin (Conget and Minguell, 1999). Laminin is bound by several integrin receptors, all of which are found in hMSCs, suggesting that laminin might play an important role in adhesion of hMSCs to PEGDA SPHs and their end fate. Integrin binding and expression relates to differential expression of ECM molecules (Grayson et al., 2004) and the differential binding of serum proteins in the SPH is expected to result in the downstream ECM component differences and possibly different populations of hMSCs being retained.

The cell-secreted ECM resembled the composition of marrow-derived ECM. It has been shown that marrow stem cell-derived ECM restrains osteoblast differentiation and promotes increased replication of multipotent colony forming units (MCFUs) (Chen et al., 2007). The ECM regulates the balance between stem cell replication and differentiation in response to appropriate stimuli. After three weeks of culture within PEGDA SPHs, hMSCs expressed CD105, CD90, CD73 and CD44 to a similar level than their counterparts grown on 2D TCP suggesting that the cells retained their multilineage differentiation potential (**Figure 2.5**).

MSCs are defined by the expression of those cell surface markers together with the ability to differentiate into downstream lineages such as adipocytes, chondrocytes and osteoblasts (Jones et al., 2002; Pittenger et al., 1999). The CD105 expression is lost when hMSCs start differentiating towards lineages of the mesenchyme. The expression of CD105 in umbilical cord blood derived hMSCs was significantly decreased in differentiated osteoblasts, chondrocytes and adipocytes from $99.4\% \pm 0.1\%$ to $3.5\% \pm 1.4\%$, $3.5\% \pm 2.3\%$ and $16.7\% \pm 3.6\%$, respectively (Kern et al., 2006). It must be noted, that there are currently no cell surface markers that are specifically and uniquely characteristic of hMSCs, for example CD105 is also associated with vascular endothelial cells (Cheifetz et al., 1992).

Variations in culture methods and differentiation stage of cells can lead to diverging results in surface marker characteristics among the reported studies. For each of the donors, similar marker distribution was identified in the SPHs, suggesting that cell populations obtained from within the hydrogels were consistent between experiments. In our experiments CD105 expression within unsorted bone marrow derived hMSCs was about $78.4\% \pm 1.2\%$ in passage 2 cells. We used passage 4 cells for comparing the surface marker expression of hMSCs cultured within PEGDA SPHs and on 2D TCP for 21 days. Since there was no significant difference in the CD105 expression between 3D scaffold ($72.5\% \pm 6.9\%$) and 2D monolayer culture ($77.5\% \pm 5\%$), it suggested that the hMSCs did not undergo substantial differentiation within the PEGDA SPHs and maintain their multi-lineage potential, but it is imperative to show this multipotent differentiation.

The true assessment of the multipotent state is the ability of the hMSCs to differentiate into specific cell types (Jones et al., 2002; Keskar et al., 2009c; Pittenger et al., 1999). The multi-lineage differentiation capability of hMSCs seeded within PEGDA SPHs was confirmed by

their ability to express adipogenic, chondrogenic and osteogenic markers (**Figure 2.6**). While there was no significant increase in the gene expression of osteogenic markers (**Figure 2.6h,i**), we observed significantly increased Ca^{2+}-levels after osteogenic induction. The discrepancies between mineralization results and the expression of osteogenic genes have been shown before on 2D TCP (Shafiee et al., 2011b). In addition to soluble factors, the stiffness of the surrounding material has been shown to guide lineage commitment of hMSCs. Softer matrices with an elastic modulus of ~ 1 kPa promoted neurogenic commitment whereas stiffer matrices with an elastic modulus of ~ 40 kPa resulted in osteogenic differentiation of MSCs (Engler et al., 2006). Preliminary data suggest a compressive modulus of about 150 kPa for PEGDA SPHs. We have not been able to determine the matrix elasticity of SPHs yet. However, for many materials the compressive modulus is similar to the elastic modulus and thus, it is suggested that PEGDA SPHs may be "stiff enough" to promote osteogenic differentiation. Yet, it also must be considered that the mechanical properties of the bulk hydrogel might not represent what the cells actually "sense" when seeded into the porous hydrogel network.

Since there is no clear evidence that all cells differentiate, future investigations into the efficiency of differentiation are warranted. The unaltered expression of stem cell surface markers along with the multi-lineage differentiation capability confirms that the cells remain multipotent mesenchymal stem cells within PEGDA SPHs under long-term culture conditions suggesting that the SPH has appropriate chemical (protein absorption) and physical characteristics for stem cell growth and maintenance. We believe that SPH structure, chemistry, and mechanics along with the specific microenvironment created by the stem cells themselves may provide biophysical cues that enable hMSCs to retain their multilineage

potency within the SPH. We are the first ones that demonstrated the presence and the development of cell-matrix interactions within unmodified macroporous PEGDA hydrogels.

2.5 Conclusions

Tissues are constructed of cells that are embedded in a dense ECM. Thus, a scaffold that enables ECM secretion recreates a major component of the native environment of cells *in vitro.* Within the PEGDA SPHs, initial hMSCs attachment is serum-dependent while survival is serum independent. Additionally, hMSCs secrete ECM that provides environmental cues for cell survival, proliferation and differentiation. In addition to enabling stem cell viability, the PEGDA SPHs supported MSC survival in an undifferentiated state while retaining the multilineage potential of the cells. Thus, the PEGDA SPH provides an opportunity for three-dimensional culture of viable and functional hMSCs under controlled conditions for stem cell maintenance and differentiation. These scaffolds have great potential not only in regenerative medicine but also as model 3-D systems for studying cell behavior in response to various stimuli.

Table 2.1 Genes and primers used for real-time PCR.

Gene	Full Name	Sequences 5´-> 3´	Accession number/ Reference
ADIPOQ	Adiponectin	For: AGG GTG AGA AAG GAG ATC C Rev: GGC ATG TTG GGG ATA GTA A	NM_004797
ACAN	Aggrecan	For: TTC AGT GGC CTA CCA AGT GGC ATA Rev: AGC CTG GGT TAC AGA TTC CAC CAA	NM_001135
ALPL	Alkaline Phosphatase	For: ATT TCT CTT GGG CAG GCA GAG AGT Rev: ATC CAG AAT GTT CCA CGG AGG CTT	NM_000478
BGLAP	Bone gamma-carboxy-glutamate (Osteocalcin)	For: CAG CGA GGT AGT GAA GAG AC Rev: TGA AAG CCG ATG TGG TCA G	NM_199173
COL1A1	Collagen type I	For: CGC TAC TAC CGG GCT GAT GAT Rev: ATC TTG AGG TCA CGG CAG GTG	NM_000088
COL2A1	Collagen type II	For: GTT GCA AAC CCA AAG GAC CCA AGT Rev: ACA TCA GGT CAG GTC AGC CAT TCA	NM_001844
COL4A1	Collagen type IV	For: ACT CTT TTG TGA TGC ACA CCA Rev: AAG CTG TAA GCG TTT GCG TA	NM_001845 (Stockert et al., 2011)
FN1	Fibronectin	For: GGT GAC ACT TAT GAG CGT CCT AAA Rev: AAC ATG TAA CCA CCA GTC TCA TGT G	NM_054034, RTPrimer DB, ID:1092 (Pattyn et al., 2006)
LAMA5	Laminin	For: CCC ACC GAG GAC CTT TAC TGC Rev: GGT GTG CCT TGT TGC TGT TGG	NM_005560 (Van Landeghem et al., 2009)
LEP	Leptin	For: CTG ATG CTT TGC TTC AAA TCC A Rev: GCT TTC AGC CCT TTG CGT T	NM_000230 (Donzelli et al., 2011)
PCNA	Proliferating cell nuclear antigen	For: AGG CAC TCA AGG ACC TCA TCA Rev: GAG TCC ATG CTC TGC AGG TTT	NM_002592
RPL13 α	Ribosomal protein L13 α	For: CAT AGG AAG CTG GGA GCA AG Rev: GCC CTC CAA TCA GTC TTC TG	NM_012423 (Curtis et al., 2010a)

Table 2.2 Flow cytometric comparison of hMSC surface markers of cells cultured within SPHs or on 2D monolayer for 21 days.

Surface marker	2D (%)[†]	SPH (%)[†]
CD105	77.5 ± 5.0	72.5 ± 6.9
CD90	99.2 ± 1.0	97.5 ± 2.6
CD73	99.2 ± 0.8	98.2 ± 2.1
CD44	99.5 ± 0.3	98.8 ± 0.3

[†]The values listed in the table are presented as the mean values of 3 different donors expressing the indicated cell surface protein ± standard deviation. There is statistical difference between the CD markers ($p < 0.001$), but no difference between the 2D and SPH samples ($p = 0.14$).
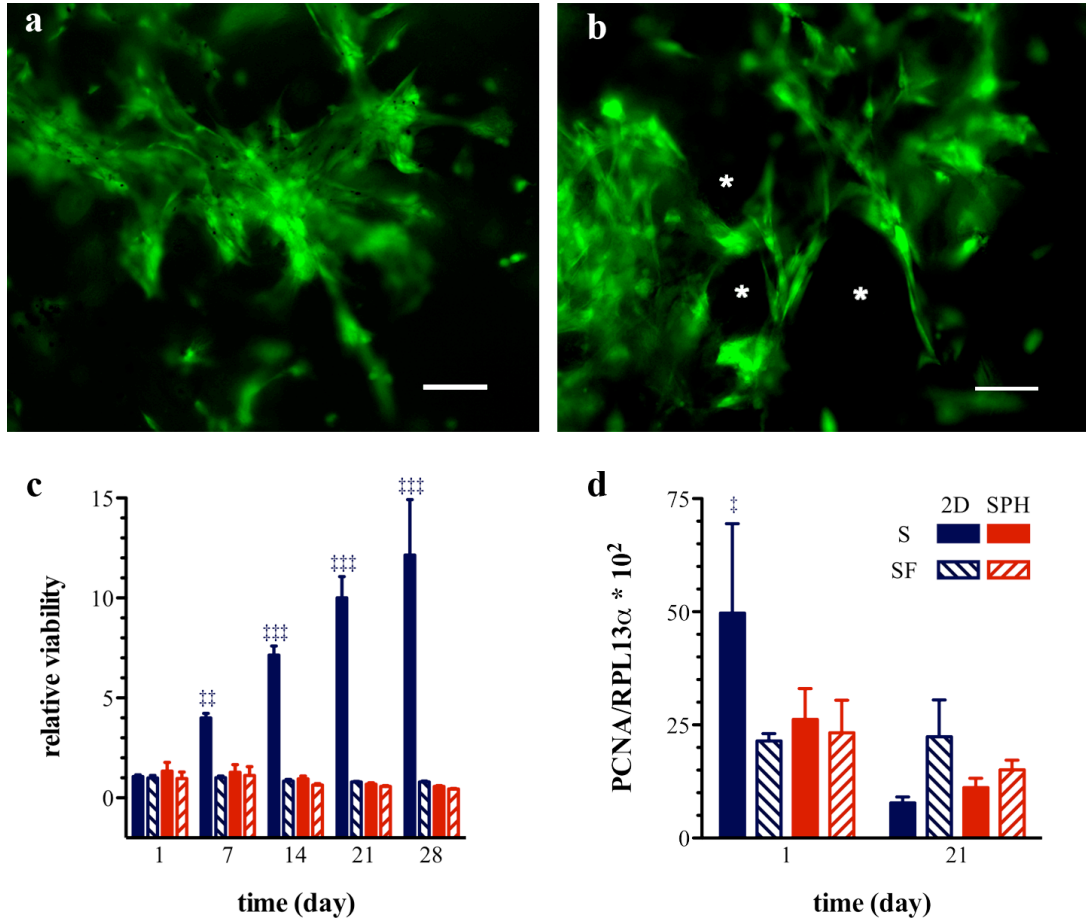
Figure 2.1 Human mesenchymal stem cells remain viable and proliferate within PEGDA superporous hydrogels. Representative pseudocolored epifluorescent micrographs of viable hMSCs around the pores (*) of PEGDA SPHs, (a) 3 and (b) 7 weeks post seeding. Viable cells are stained with calcein acetoxymethyl ester dye (green) and dead cells appear red due to staining with ethidium homodimer. The scale bar is 100 μm. (c) MSC viability within PEGDA superporous hydrogels (SPH/red) and on tissue culture plastic as a monolayer (2D/blue) under serum-containing (S/solid bars) and serum-free conditions (SF/diagonal lines within bars) was assessed by the MTS assay. Cells were incubated for one day in the presence of serum, at t = 0 day, and maintained for additional time (as listed on x-axis) in either serum-containing (S) or serum-free conditions (SF). The symbols (‡‡) and (‡‡‡) denote a significant higher cell viability under 2D culture conditions in the presence of serum compared to all other groups at p < 0.01 and p < 0.001 respectively. (d) The proliferative activity of hMSCs was evaluated by the gene expression of PCNA. The symbol (‡) denotes a significant difference in the PCNA mRNA expression at day 1 under 2D culture conditions in the presence of serum compared to all other groups at p < 0.05 (n=3-4; mean ± standard deviation).
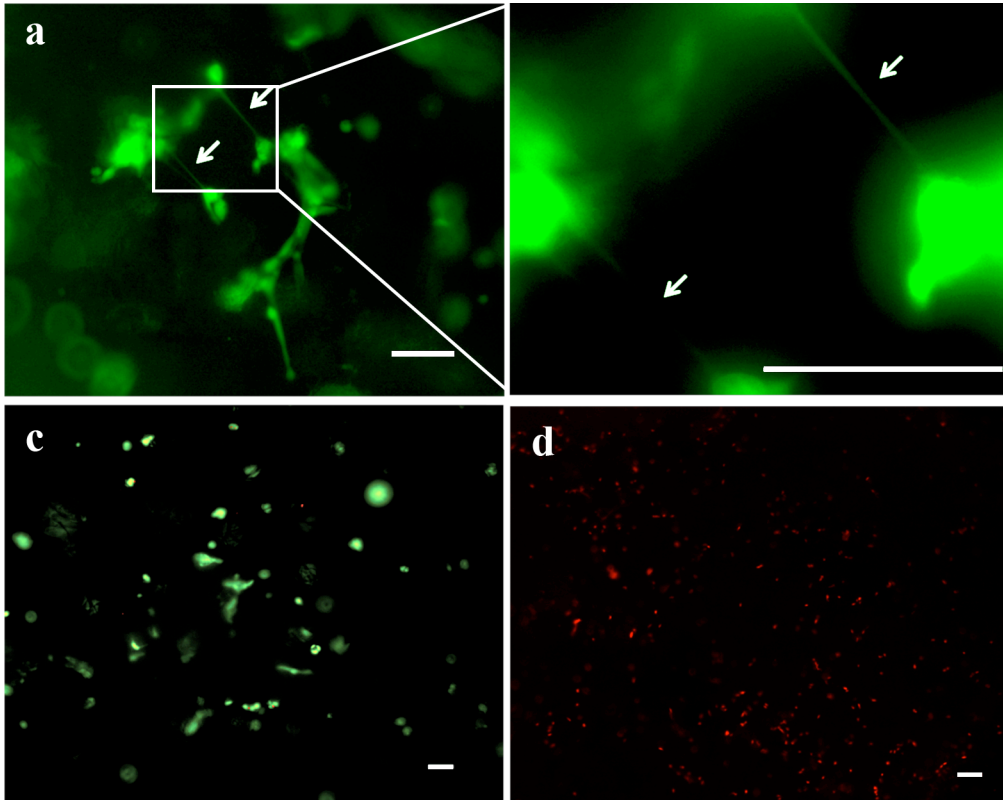
Figure 2.2 Serum proteins are required for initial anchorage of human mesenchymal stem cells to PEGDA superporous hydrogels. Representative live (green)-dead (red) pseudocolor stained images for hMSCs seeded within PEGDA SPHs and cultured in complete DMEM for 24 hours. (a) Cells with extended morphology toward nearby cells as marked by arrows could be observed. (b) Magnified region from (a) to show the cell-cell projections where the arrows are in approximately the same location. (c) When the culture was deprived of serum for the following 24 hours (total 48 hour culture), the cells remained viable. (d) However, when the hMSCs were seeded in PEGDA SPHs and cultured directly in serum-free medium for 24 hours, the cells did not survive (red staining), as confirmed by the absence of live cells (no green) within the live-dead staining image. The scale bar is 100 μm in each image. *Picture was kindly provided by Dr. Vandana Keskar*.
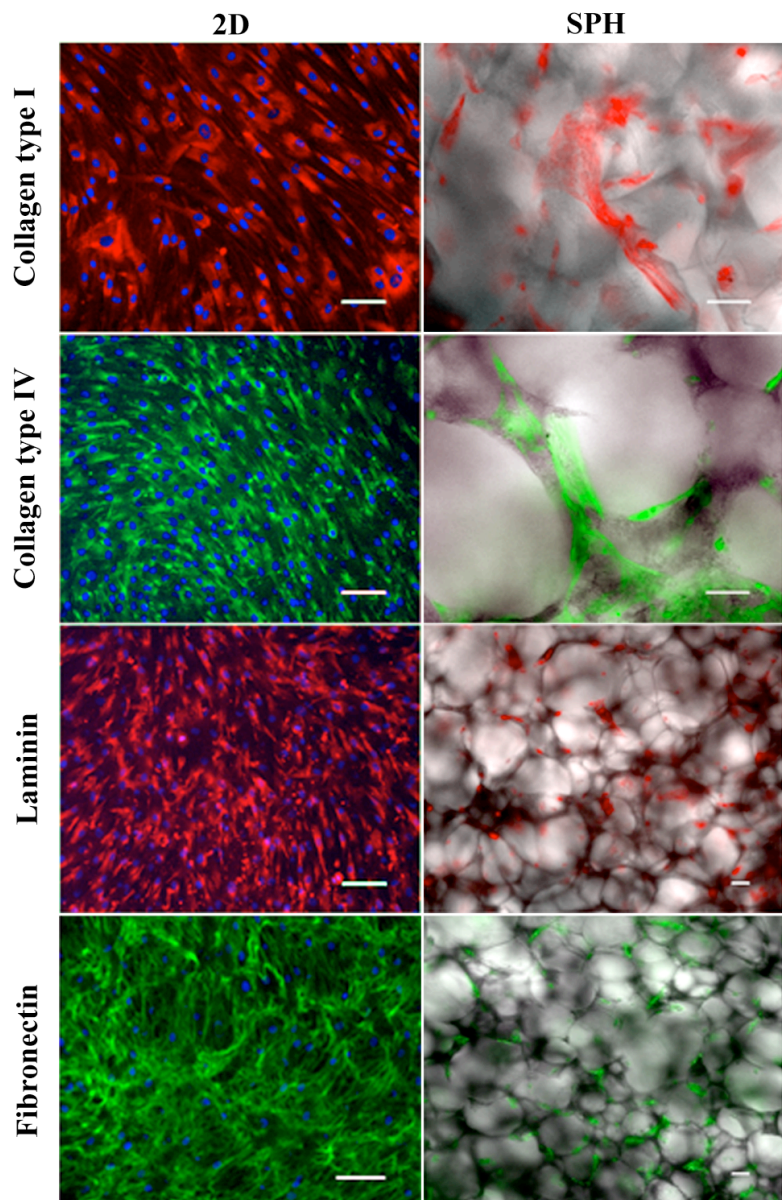
Figure 2.3 Human mesenchymal stem cells synthesize their own extracellular matrix proteins when cultured within PEGDA superporous hydrogels. Representative pseudocolored immunohistofluorescence micrographs of collagen type I, collagen type IV, fibronectin and laminin expressed by hMSCs cultured within SPHs and on tissue culture plastic (TCP) for 21 days. The expression of the four ECM proteins was positive in both culture systems. H33258 (blue) was used as a nuclear stain in the monolayer group. Due to strong background staining no H33258 staining was conducted in the SPH group. Scale bar is 100 μm in each image. *Picture was kindly provided by Dr. Vandana Keskar.*
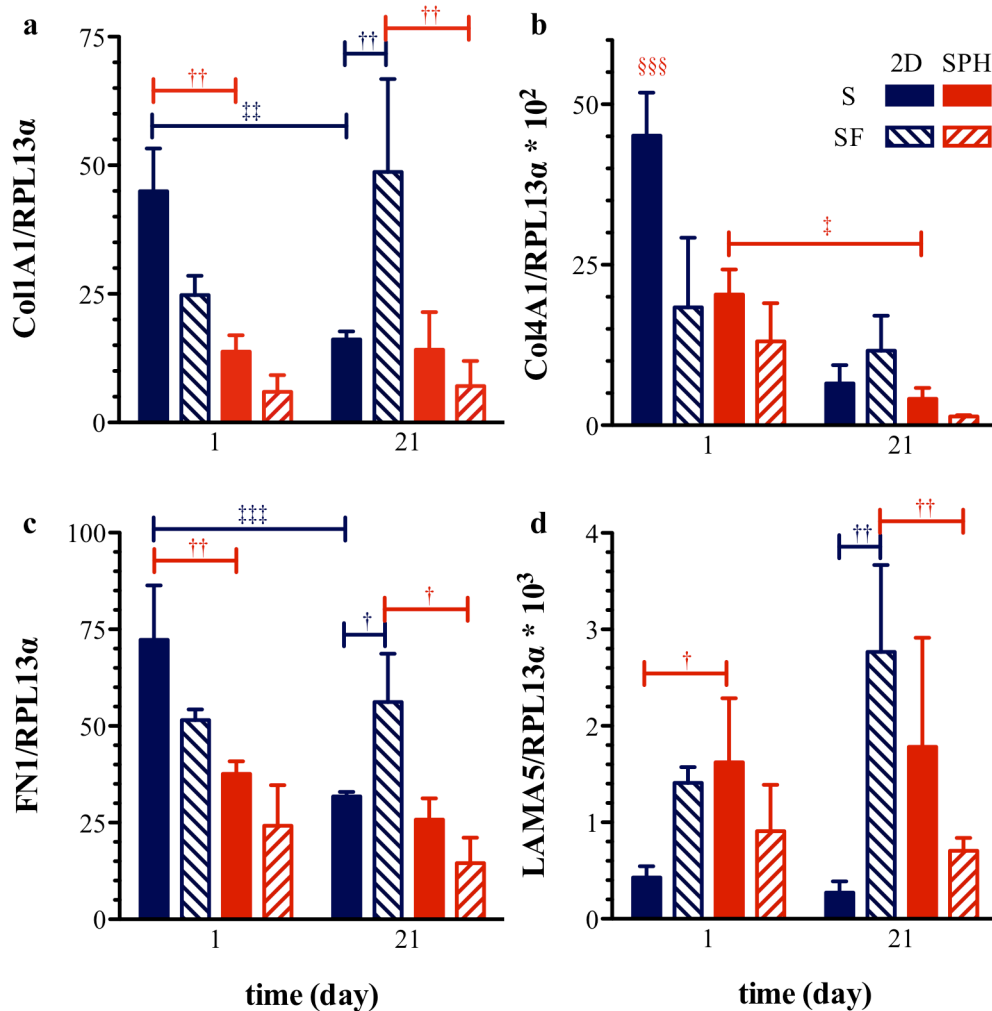
Figure 2.4 Expression profiles of genes encoding for extracellular matrix proteins. Expression of (a) collagen type I, (b) collagen type IV, (c) fibronectin, and (d) laminin in hMSCs cultured for 1 and 21 days within PEGDA superporous hydrogels (SPH/red) and on tissue culture plastic as a monolayer (2D/blue) under serum-containing (S/solid bars) and serum-free conditions (SF/diagonal lines within bars); mRNA levels were normalized to the expression of the endogenous control ribosomal protein L13 α (*RPL13α*). Collagen 1A1 (*Col1A1),* collagen 4A1 *(Col4A1)* and fibronectin (*FN1)* expression was similar to 2D monolayer within the PEGDA SPH system after 21 days of culture in serum-containing basal medium. Values are presented as mean ± standard deviation (n=3-4). Statistical significance is indicated in the figure as † for differences within a specific day, ‡ for differences between day 1 and 21, and § for difference from all other groups on day 1 and the number of symbols indicating level of significance with one, two and three symbols indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.
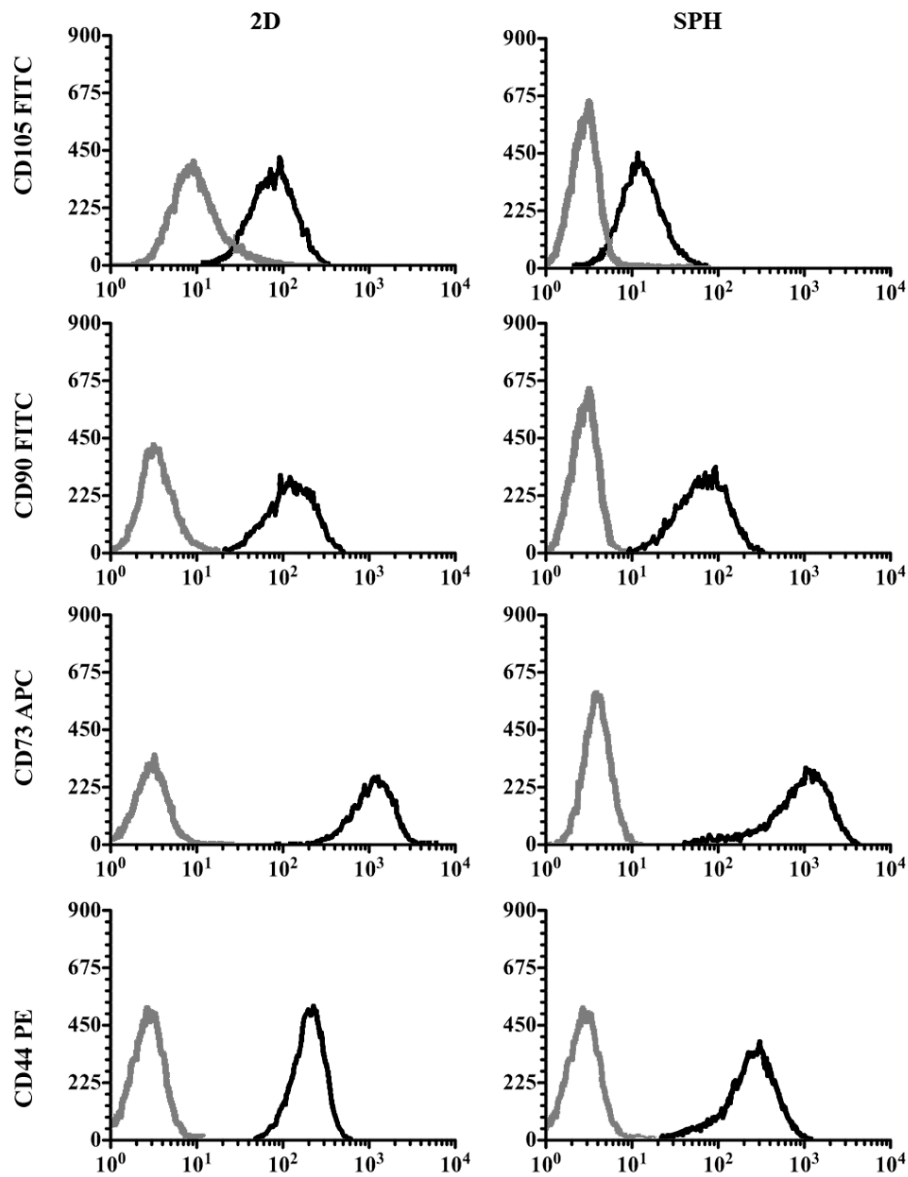
Figure 2.5 Expression of human mesenchymal stem cell surface markers of cells cultured within PEGDA superporous hydrogels or on tissue culture plastic (TCP) for 21 days. Representative flow cytometry histograms from three independent experiments with three different donors are shown. Analysis revealed no statistically significant differences in the expression of CD105, CD90, CD73 and CD44 in hMSCs cultured within SPHs (right) or in 2D (left). Gray lines represent the fluorochrome- and isotype-matched control and black lines the corresponding CD marker-specific antibody.
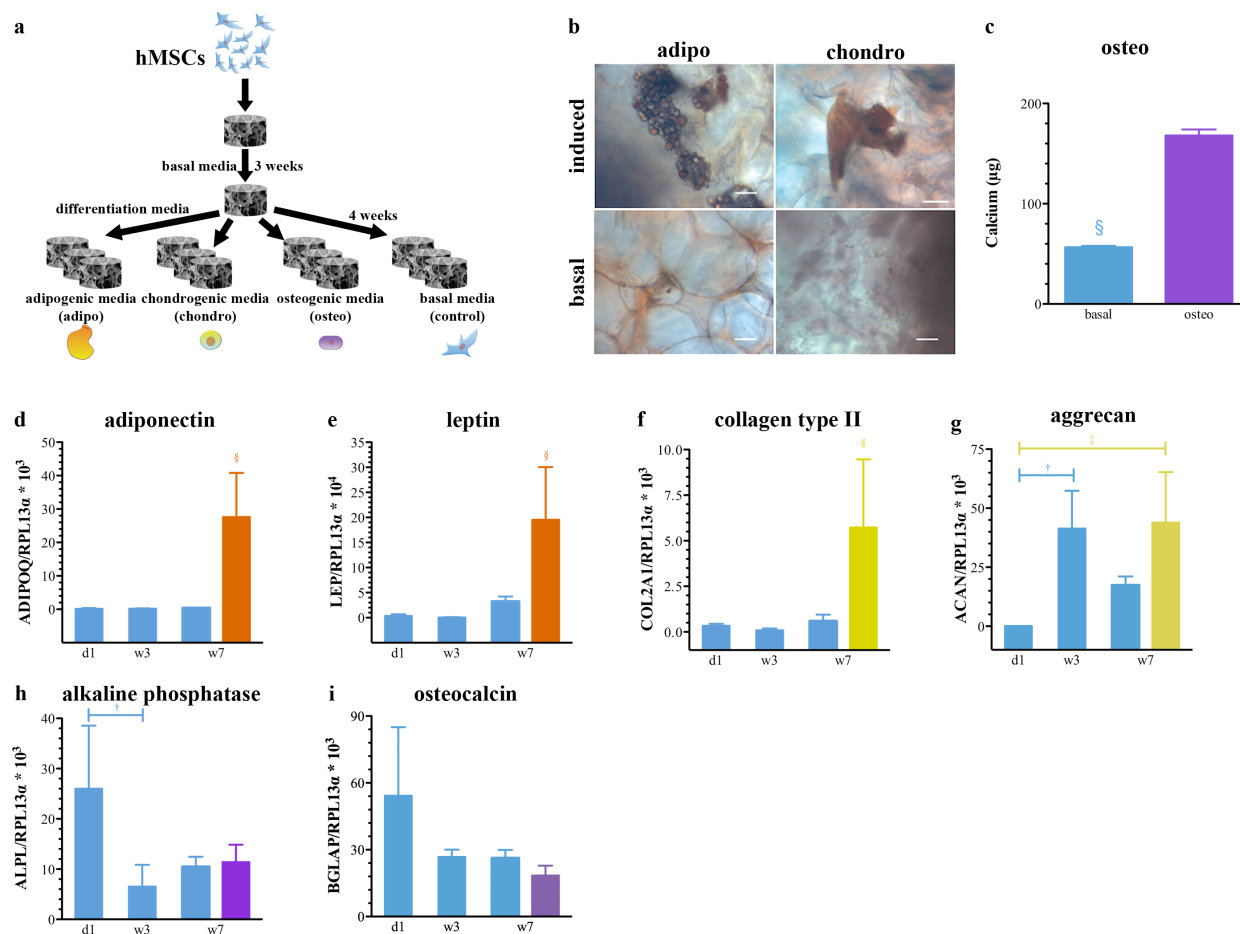
Figure 2.6 Human mesenchymal stem cells retain their multilineage differentiation capability within superporous hydrogels. (a) Schematic of treatment regimen for cell-seeded constructs cultured for 3 weeks in basal medium and additional 4 weeks in adipogenic, chondrogenic, and osteogenic differentiation medium. Controls were maintained in basal medium for the entire culture time of 7 weeks. (b) Upon adipogenic induction, lipid vacuoles stained red with Sudan III could be observed throughout the SPH. No lipid vacuoles could be detected within the non-induced controls. (b) The induction of chondrogenesis was confirmed by the presence of proteoglycans (red) visualized by a Safranin O stain. No positive staining for proteoglycans could be detected within the basal group. Scale bars are 100 µm. (c) Osteogenic differentiation of hMSCs within SPHs was evaluated by assaying the calcium content within the mineralized matrix. Relative expression of fat-related genes (d-e), cartilage-related genes (f-g) and bone-related genes (h-i) in hMSCs cultured within PEGDA SPHs for 1 day (d1), 3 weeks (w3) and 7 weeks (w7) under basal (blue) culture conditions or for 3 weeks under basal conditions with additional 4 weeks in adipogenic (w7 orange), chondrogenic (w7 yellow) and osteogenic (w7 purple) induction medium. Statistical significance is indicated in the figure as † for differences within the basal groups, ‡ for differences between basal and differentiation media, and § for difference from all other groups at p < 0.05. Values are presented as mean ± standard deviation (n=3, 3 donors).

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3 Markers Are Shared Between Adipogenic and Osteogenic Differentiated Mesenchymal Stem Cells

3.1 Introduction

The human bone marrow stroma contains multipotent mesenchymal cells that give rise to adipocytes and osteoblasts, as well as many other lineages (Caplan and Dennis, 2006a). Cells isolated based on adherence to the tissue culture substrate do not represent a homogenous population of mesenchymal progenitors rather subpopulations of cells with variable differentiation potential (Muraglia et al., 2000; Pittenger et al., 1999). Most of the clones derived from bone marrow stromal cells possess osteogenic and adipogenic differentiation potential but some are only able to differentiate towards osteoblasts. The mechanisms of the differentiation process from precursor to fully differentiated mature cells are still not fully understood (Discher et al., 2009; Hwang et al., 2008; Scadden, 2006). Methods have been developed for differentiating cells into specific differentiated cell types expressing the markers and phenotypes of the desired tissues (Pittenger et al., 1999). New materials-based and soluble factor-based differentiation protocols are constantly being developed to control the differentiation potential of all stem cell types (Fekete et al., 2012; Hoshiba et al., 2012; Keskar et al., 2009e; Kollmer et al., 2012; Vater et al., 2011b). Many of these protocols are validated to confirm the presence of specific differentiation markers, but frequently alternative differentiation pathways are not excluded. In addition, many in the field of tissue engineering utilize a small subset of markers (Bakhshandeh et al., 2012; Choi et al., 2010; He

et al., 2012; Henderson et al., 2008; Hess et al., 2012; Marion et al., 2006; Pountos et al., 2007; Wiren et al., 2011; Zhang et al., 2012) despite the potential for expression of these markers in other differentiated lineages.

There are several examples in the literature showing the expression of a differentiation marker by multiple cell types. Leptin, an adipokine produced by adipocytes was observed on the mRNA level in human osteoblasts during the mineralization period (Reseland et al., 2001) as well as in hMSCs that underwent osteogenesis (Noh, 2012). Leptin has pleiotropic effects on other bone marrow cells, including osteoblasts (Noh, 2012; Nuttall and Gimble, 2004) and was shown to promote osteogenesis and to inhibit adipogenesis in immortalized human marrow stromal cells (Thomas et al., 1999). This could be paracrine communication controlling the growth and differentiation of adipocytes and signaling osteogenesis when sufficient adipocytes are present.

Alkaline phosphatase, widely used as a biochemical marker of bone turnover, also plays a role in adipogenesis. Inhibition of tissue-nonspecific alkaline phosphatase resulted in a decreased accumulation of lipid vacuoles during adipogenic differentiation of a murine preadipocyte cell line (Ali et al., 2005). Similarly, osteopontin (OPN) is not solely a key regulator of bone development, rather a multifunctional extracellular matrix (ECM) associated protein involved in inflammatory processes, tumorigenesis, cardiac fibrosis and obesity (Sodek et al., 2000). Upregulated OPN mRNA levels have been detected in adipose tissue of obese patients (Chapman et al., 2010). Osteocalcin, a non-collagenous protein found in mineralized adult bone, is another widely used bone marker. However, constitutive osteocalcin mRNA and protein expression by adipose stromal cells implicates that nonosteogenic cells of the marrow stroma also secrete osteocalcin (Benayahu et al., 1997).

Exposure to the glucocorticoid dexamethasone which is a constituent of both, osteogenic and adipogenic differentiation media, has been shown to increase osteocalcin expression in cultured stromal cells (Leboy et al., 1991).

We can infer from these reports that those markers need further validation as tissue-specific differentiation markers. In the present study, we assessed the suitability of fatty acid binding protein 4, adiponectin and leptin as adipogenic differentiation markers and alkaline phosphatase, collagen type I, osteocalcin and osteopontin as osteogenic differentiation markers by evaluating the expression of these markers during adipogenic and osteogenic culture conditions. We show that these markers are not selectively expressed when cells are differentiated using common differentiation protocols.

3.2 Materials and Methods

3.2.1 MSC isolation and differentiation

Human bone marrow aspirates were obtained from AllCells, LLC (Emeryville, CA) and isolated by density gradient centrifugation utilizing Ficoll-Paque^{TM} PLUS solution followed by cell-surface marker negative selection with RosetteSep$^{®}$ Human Mesenchymal Stem Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. For each experiment, hMSCs isolated from one of three donors (non-smoker males ranging in age from 20 to 31 years old) were used with no cells used beyond passage four. Cells were harvested using 0.25% trypsin with 1.0 M EDTA, centrifuged, and expanded in basal medium which consists of high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 unit/mL streptomycin (basal medium). Medium was changed every third day. Adipogenic differentiation was initiated by culturing 2 x 10^5 hMSCs in a

well of a 6 well plate in MesenCult® adipogenic induction medium (Stem Cell Technologies, Vancouver, BC, Canada). The composition of the adipogenic medium is proprietary. Adipogenic differentiation protocols routinely involve combinations of dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), insulin, and indomethacin (Vater et al., 2011b). Osteogenic differentiation was initiated by culturing 3 x 10^4 hMSCs in a well of a 6 well plate in Poietics^{TM} osteogenic induction medium (Lonza, Walkersville, MD, USA) containing dexamethasone, ascorbic acid and β-glycerophosphate supplements (Pittenger et al., 1999; Vater et al., 2011b). Controls were maintained in basal medium for the entire culture period of 4 weeks.

3.2.2 Quantitative real-time PCR

After 14 and 28 days, total RNA was extracted using the TRIzol® reagent (Life Technologies, Grand Island, NY, USA) in combination with the PureLink^{TM} RNA Mini Kit (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. To diminish genomic DNA contamination, RNA was treated with Turbo^{TM} DNAse (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. The purified RNA (10 ng/mL) was reverse transcribed with the High Capacity cDNA Reverse Transcription (RT) Kit (Life Technologies, Grand Island, NY, USA) under the following conditions: 25°C for 10 min, 37°C for 120 min followed by 85°C for 5 min. To identify potential genomic DNA contamination, controls with no enzyme were evaluated. The PCR reactions were performed on an Applied Biosystems StepOnePlus^{TM} PCR machine using 5 µL SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY, USA), 2 µL sequence specific primers (0.5mM, *GAPDH* was used at 0.25 mM, **Table 3.1**) and 3 µL cDNA (cDNA dilutions: *ADIPQ, ALPL, FABP4, OPN*: 10 fold*, BGLAP*: 5 fold*, COL1A1,*

LEP: 31 fold) under the following conditions: 95°C for 10 min followed by 40 cycles of 15 s of denaturation at 95ºC and 60 s of annealing and elongation at 60ºC. A melting curve analysis was performed after each run to confirm product specificity. The delta-delta-Ct method (Livak and Schmittgen, 2001) was employed to determine the relative gene expression level of the gene of interest normalized to the endogenous controls glyceraldehyde-3-phosphate (*GAPDH*) and ribosomal protein L13A (*RPL13A*). Statistical significance was determined using first a two-way ANOVA comparing the treatments and time followed by Newman-Keuls' post-hoc comparison of groups.

3.2.3 Immunofluorescence

Briefly, the samples were fixed in 4% paraformaldehyde, rinsed with 1X PBS and incubated with blocking solution (1% BSA in 1X PBS) for 30 min on a shaker. Osteocalcin mouse monoclonal IgG antibody (25µL; 100 µg/mL; Santa Cruz Biotech, Santa Cruz, CA, USA) was added directly to 500 µL blocking solution and samples were further incubated for 3 hours at room temperature (RT). Samples were washed with 1X PBS and incubated with 5 µL AlexaFluor® 488-labeled goat anti-mouse secondary antibody (2 mg/mL; Molecular Probes, Carlsbad, CA) in 500 µL 1X PBS at RT and protected from light. After 25 min, 0.5 µL H33258 (1 mg/mL; Life Technologies, Grand Island, NY, USA) was added to the solution and incubated for an additional 5 min at RT. The secondary antibody solution was discarded after 30 min and the samples were washed with 1X PBS. Images were taken with an Olympus IX70 inverted microscope and processed using QCapture Pro software.

3.2.4 Sudan III staining

To stain lipid vacuoles, the samples were rinsed with 1X PBS (pH 7.4) and incubated with Sudan III solution (0.3% w/v of Sudan III in 70% ethanol) for 3 minutes. After several

washes with double deionized water (DDIW), Harris hematoxylin solution was added and incubated for 1 minute. Samples were destained in fresh acid ethanol (0.5% 1 N HCL in 70% EtOH) for 1 min. Afterwards the wells were rinsed with DDIW until the water ran clear. Images were taken under bright field with an Olympus IX70 inverted microscope.

3.3 Results

Depending on the reference genes, sometimes referred to as housekeeping genes, used in the qPCR analysis, different gene expression patterns have been observed during osteogenic differentiation (Quiroz et al., 2010). Instabilities in gene expression levels during cell differentiation must be taken into account. The genes of interest were normalized to the most commonly used reference genes, *GAPDH* and *RPL13A*. These reference genes were also chosen based upon their recent assessment as reference genes in hMSCs (Curtis et al., 2010b).

When normalized against *GAPDH*, there is significant variation in the results and two of the three genes examined lacked statistical significant (**Figure 3.1A, 3.1C, 3.1E**). When normalized against *RPL13A*, adipocyte differentiation markers (*ADIPQ*, *FABP4*; *LEP*) were significantly upregulated ($P < 0.5$) under adipogenic conditions at day 28 (**Figure 3.1B, 3.1D, 3.1F**). Consistent with the postulate of an inverse relationship between adipo- and osteogenesis (Nuttall and Gimble, 2004), adipocyte-associated genes were not significantly upregulated in osteogenic differentiation groups. *ADIPQ* and *LEP* were still detectable in cells that underwent osteogenic differentiation (**Figure 3.1B, 3.1C, 3.1D, 3.1F**), but at a much lower level. Lipid vacuoles that represent the adipogenic phenotype were only present in the adipogenic induction group (**Figure 3.2**). No lipid vacuoles could be detected in the osteogenic induction groups at any time point. In contrast, osteogenic genes were upregulated

during adipogenesis. With both reference genes, alkaline phosphatase (*ALPL*) mRNA levels were upregulated under adipogenic and osteogenic differentiation conditions (**Figure 3.1G, 3.1H**). Significantly elevated osteopontin (*OPN*) mRNA levels ($P < 0.5$) were observed in cells that were cultured in adipogenic medium for 28 days compared to the non-induced control and the osteogenic induction group when *OPN* was normalized against *RPL13A* (**Figure 3.1N**). At day 14, *OPN* levels were higher in the osteogenic induction group. Early bone marker, collagen type I (*COL1A1*), mRNA levels were significantly elevated ($P < 0.5$) in the osteogenic induction group compared to the adipogenic group at day 14 when normalized to *GAPDH* (**Figure 3.1K**). Due to variations between donors, no statistically significant differences in the expression of the late osteoblast marker osteocalcin (*BGLAP*) were observed between adipogenic and osteogenic treatment groups (**Figure 3.1I, 3.1J**). Similar to the *COL1A1* expression pattern*, BGLAP* appears to be expressed in adipocyte cultures at day 14. Interestingly, *BGLAP* was significantly upregulated under basal conditions at day 28 when normalized to *GAPDH* (**Figure 3.1I**). Quiroz *et al.* obtained similar results due to the high variability of *GAPDH* under basal culture conditions (Quiroz et al., 2010). The relative quantification with *RPL13A* seemed to produce lower standard deviations in the expression levels of adipogenic genes and thus gave more significance to the results. The overall expression patterns of adipogenic and osteogenic genes were similar with both housekeeping genes.

Immunostaining was carried out to detect and visualize the expression of osteocalcin (**Figure 3.3**). Similar to the mRNA expression data, osteocalcin was detected in the adipogenic induction group as well as in the osteogenic induction group at both time points (**Figure 3.3**). Under both conditions, osteocalcin expression levels decreased from day 14 to

day 28. In contrast to the mRNA data, no osteocalcin protein expression was detected in the basal control groups.

3.4 Discussion

Dexamethasone is a mutual component of adipogenic and osteogenic induction medium and has been shown to increase osteocalcin and leptin production in hMSCs (Leboy et al., 1991; Noh, 2012). Although additional compounds are added to the differentiation cocktails heterogeneity in the cultures still exists (Pittenger et al., 1999; Vater et al., 2011b). Whether this phenomenon results from the plasticity of those cells and hMSCs that progress towards one lineage can transdifferentiate into another lineage, or whether the current set of markers is not definitive enough for one lineage, still needs further investigation. Similar to our observation, simultaneous expression of osteoblast markers (alkaline phosphatase and osteocalcin) and adipocyte markers (peroxisome proliferator-activated receptor γ 2 and lipoprotein lipase) was confirmed on a single cell level in hMSC-derived osteoblasts (Ponce et al., 2008).

When hMSCs differentiate into mature osteoblasts they have to pass through several maturation stages that are characterized by a time-dependent expression of gene markers. Alkaline phosphatase and collagen type I mRNA levels are upregulated during early stages of bone formation (Jaiswal et al., 1997; Jikko et al., 1999). Osteopontin expression peaks twice during bone development, in the proliferation phase (~ day 4) and in the mineralization phase (~ day 14-21) (Aubin, 2001). Osteocalcin and osteopontin are highly expressed during the last stage of bone formation, the mineralization period. Although commonly used as an indicator for osteogenic differentiation, almost all of these markers are not bone specific. Alkaline phosphatase, an ubiquitous cellular protein, was upregulated during adipogenic and

osteogenic differentiation (**Figure 3.1B, H**). Alkaline phosphatase does confirm initiation of differentiation but cannot be considered as lineage-specific. Collagen type I is the main component of bone extracellular matrix (ECM) but has been identified in a number of unrelated cell types (Hing, 2004). In our study, *COL1A1* expression was upregulated under osteogenic and downregulated under adipogenic conditions at day 14 (**Figure 3.1K**). At later time points, we observed no differences in the *COL1A1* expression levels between adipocyte and osteocyte cultures. Osteopontin cannot be considered as a bone-specific marker either since it regulates cell adhesion, migration and survival in other tissues as well (Sodek et al., 2000). *OPN* was detected in adipogenic and osteogenic lineages but gene expression patterns were distinct from each other with *OPN* being upregulated at earlier time points in osteoblast cultures and at later stages in adipocyte cultures (**Figure 3.1N**). In contrast to the protein levels (**Figure 3.3**), osteocalcin mRNA levels were not significantly upregulated during osteogenic induction (**Figure 3.1I, 3.1J**). We obtained similar results when hMSCs underwent osteogenic differentiation within a 3D hydrogel scaffold (Kollmer et al., 2012). Although Ca^{2+}-levels were significantly upregulated, no increase in osteogenic gene expression was observed. A discrepancy between mineralization and gene expression data indicated that an up regulation in osteogenic genes in hMSCs does not correlate with their ability to differentiate towards the osteogenic lineage (Shafiee et al., 2011a). Glucocorticoid-mediated down regulation of osteocalcin mRNA levels has also been reported (Viereck et al., 2002).

The presence of osteocalcin in hMSC-derived adipocytes is in concert with a recently published study where osteocalcin was detected in human preadipocytes and to a lesser extent in fully differentiated adipocytes (Foresta et al., 2010). Osteocalcin's role in human

physiology has been further expanded as a circulating hormone influencing beta-cell proliferation, glucose intolerance, and insulin resistance has recently arisen (Lee et al., 2007).

3.5 Conclusion

The current work and previous reports clearly indicate that many of the markers used for determining the end fate of adipocytic and osteoblastic differentiation are shared between adipogenic and osteogenic differentiated hMSCs. Adipocytes and osteoblasts share a common pool of precursor cells and their plasticity is regulated by activation or silencing of genes, signaling molecules and transcription factors (Garces et al., 1997; Gimble et al., 1996). Our data indicate the need for a better understanding of the conditions and molecular regulators involved in controlling the plasticity of hMSCs. This knowledge is a prerequisite to manipulate adult stem cells for engineering functional tissues in regenerative medicine and to shed light into the pathogenesis of metabolic and skeletal disorders, like atherogenesis, diabetes and osteoporosis. Furthermore, the present study suggests that differentiation towards one lineage should be accompanied by evidence indicating lack of differentiation into other lineages.

Table 3.1 Adipogenic and osteogenic genes and primers used for real-time PCR.

Gene	Full Name	Sequences 5´-> 3´	Accession number/ Reference
ADIPOQ	Adiponectin	For: AGG GTG AGA AAG GAG ATC C Rev: GGC ATG TTG GGG ATA GTA A	NM_004797
ALPL	Alkaline Phosphatase	For: ATT TCT CTT GGG CAG GCA GAG AGT Rev: ATC CAG AAT GTT CCA CGG AGG CTT	NM_000478.4
BGLAP	Bone gamma-carboxyglutamate (Osteocalcin)	For: CAG CGA GGT AGT GAA GAG AC Rev: TGA AAG CCG ATG TGG TCA G	NM_199173
COL1A1	Collagen type I	For: TGT GGC CCA GAA GAA CTG GTA CAT Rev: ACT GGA ATC CAT CGG TCA TGC TCT	NM_000088
FABP4	Fatty acid binding protein 4	For: TGG TTG ATT TTC CAT CCC AT Rev: TAC TGG GCC AGG AAT TTG AC	NM_001442
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	For: TTC GAC AGT CAG CCG CAT CTT CTT Rev: GCC CAA TAC GAC CAA ATC CGT TGA	NM_002046.4
LEP	Leptin	For: CTG ATG CTT TGC TTC AAA TCC A Rev: GCT TTC AGC CCT TTG CGT T	NM_000230
OPN	Osteopontin	For: AGA ATG CTG TGT CCT CTG AAG Rev; GTT CGA GTC AAT GGA GTC CTG	NM_001251830
RPL13A	Ribosomal protein L13 α	For: CAT AGG AAG CTG GGA GCA AG Rev: GCC CTC CAA TCA GTC TTC TG	NM_012423
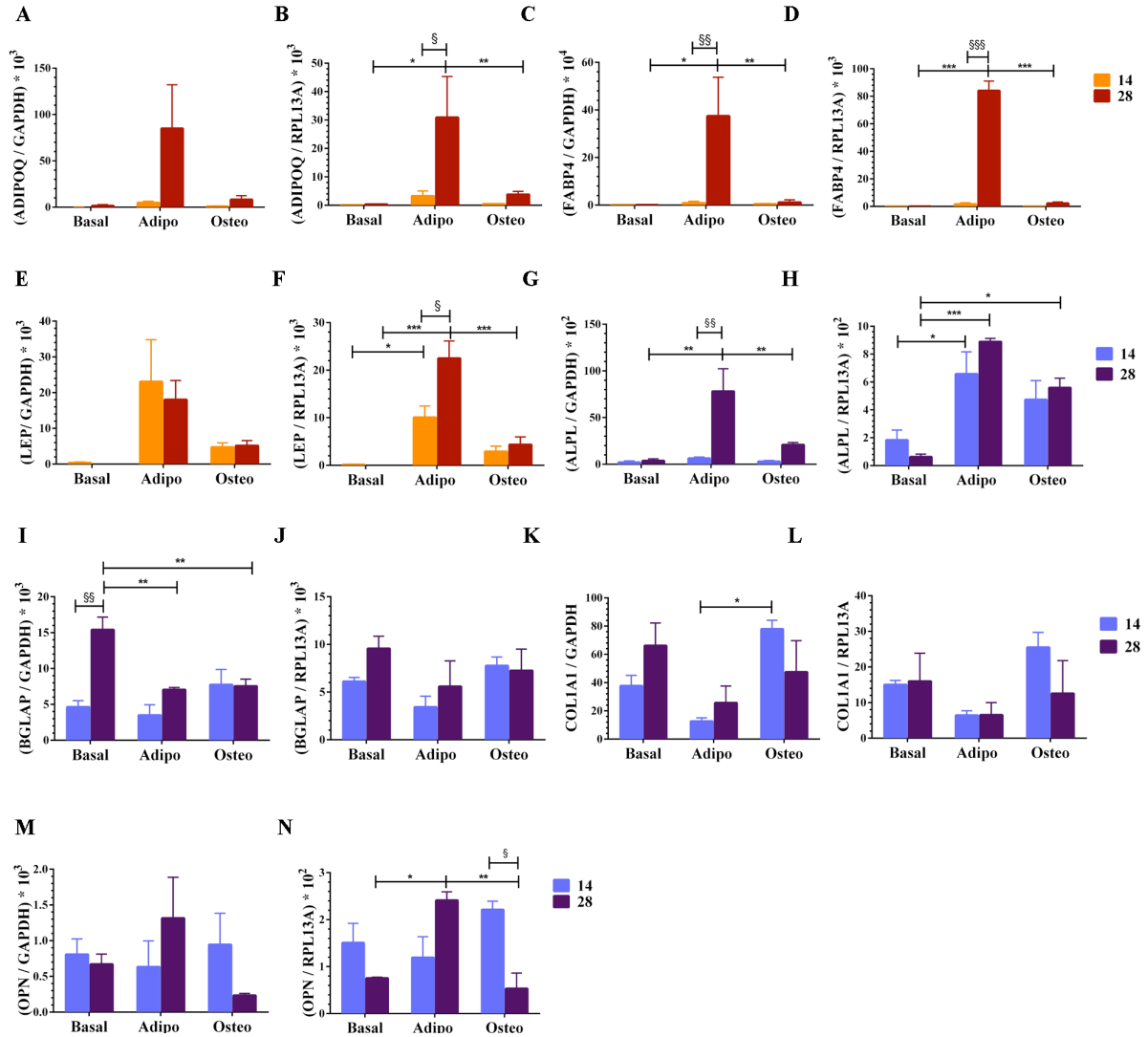
Figure 3.1 Expression profiles of genes encoding for adipocyte- and osteoblast-related genes. Expression of adipocyte marker genes (A-B) adiponectin (*ADIPQQ*), (B-C) fatty acid binding protein 4 (*FABP4*), (D-E) leptin (*LEP*), and osteoblast marker genes (G–H) alkaline phosphatase (*ALPL*), (I–J) osteocalcin (*BGLAP*), (K-L) collagen type I (*COL1A1*), (M-N) osteopontin (*OPN*) in hMSCs cultured in basal, adipogenic (adipo) and osteogenic (osteo) induction medium for 14 and 28 days; mRNA levels were normalized to the expression of endogenous control genes glyceraldehyde-3-phosphate (*GAPDH*) and ribosomal protein L13A (*RPL13A*). Values are presented as mean plus or minus (±) standard error of the mean (n=3 donors). Statistical significance is indicated as (*) for differences between treatment groups at day 14 and 28, respectively and (§) for the difference between day 14 and 28 for a given treatment and the number of symbols indicating level of significance with one, two, and three symbols indicating p < 0.05, p < 0.01, and p < 0.001, respectively.
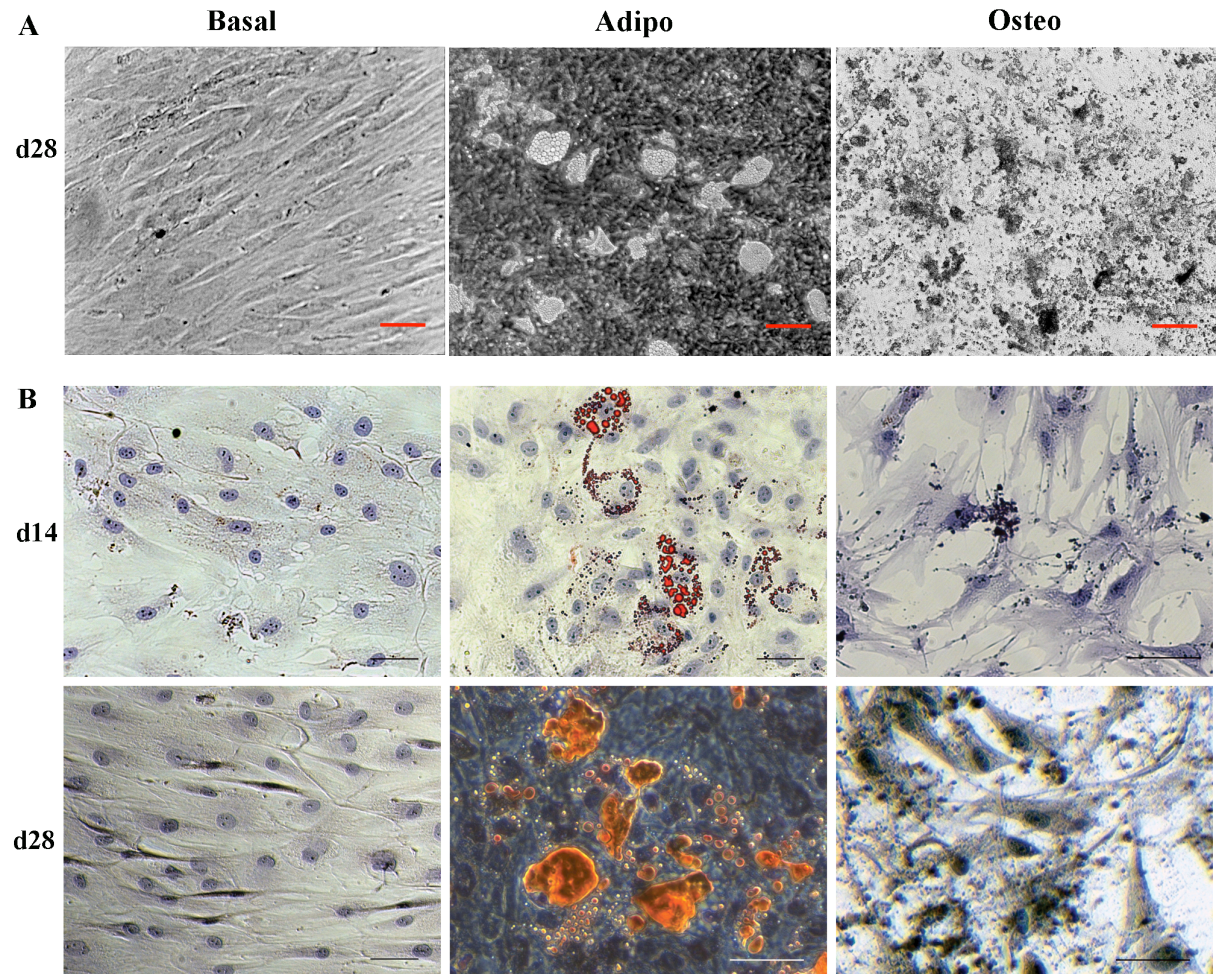
Figure 3.2 Morphology of differentiated cells. (A) Cells in the untreated control group (basal) maintained an undifferentiated phenotype with fibroblast-like cells, lipid vacuoles were visible in the adipogenic induction group (adipo) and black regions within the cell monolayer indicate calcification in the osteogenic induction group (osteo) at day 28 of differentiation. (B) To evaluate adipogenic differentiation, Sudan III was conducted. The presence of lipid vacuoles was observed in the adipo group at day 14 and 28. No lipid vacuoles could be seen in the basal and in the osteo group. The scale bar in each image is 50 μm.
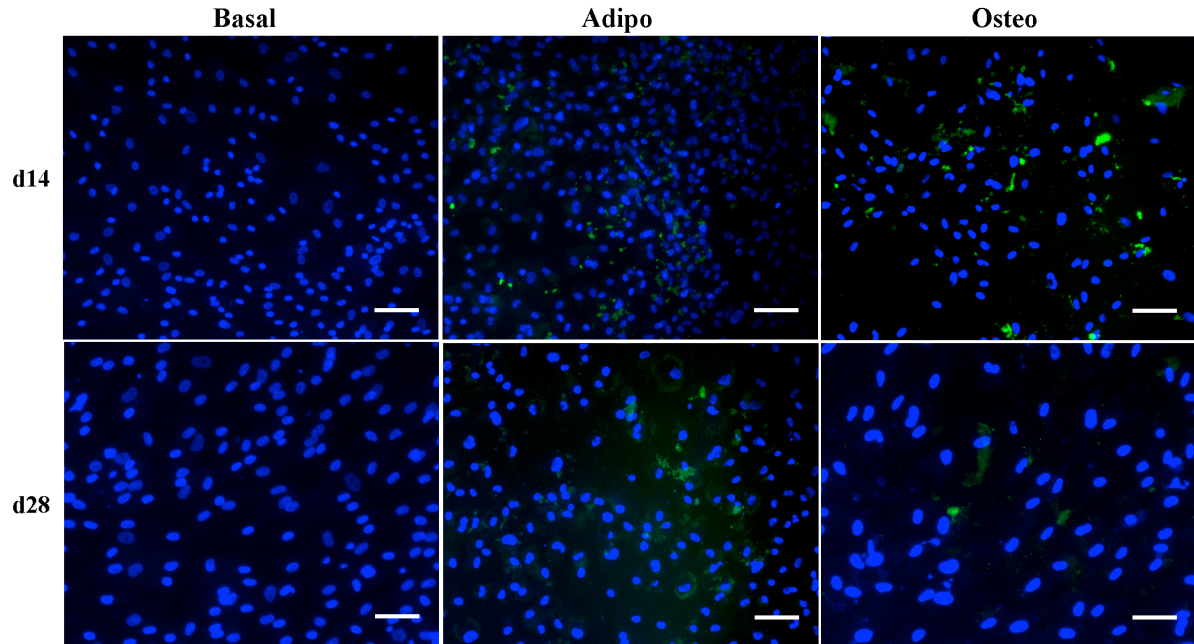
Figure 3.3 Representative immunofluorescence micrographs of osteocalcin expression in hMSCs, hMSC-derived adipocytes (adipo), and hMSC-derived osteoblasts (osteo). Osteocalcin expression (green) was detected to a comparable level in hMSC-derived adipocytes and osteoblasts. The expression in both groups decreased at day 28 compared to day 14. No osteocalcin was detected in the basal control. H33258 (blue) was used as a nuclear stain. Scale bar is 50 μm.

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4 Evaluation of Endothelial-like hMSCs for Vascularization of TE Constructs

4.1 Introduction

Tissue-specific cells are often limited in their availability and quality. Multipotent mesenchymal stem cells (MSCs) have shown increasing potential in cell-based tissue engineering (TE) due to their capability to differentiate towards adipo-, osteo- and chondrogenic lineages (Pittenger et al., 1999). It is suggested that MSCs are also involved in the generation of non-mesodermal cell types such as neurons (Tondreau et al., 2008), myocardial cells (Choi et al., 2011), endothelial-like cells (Oswald et al., 2004), and hepatocyte-like cells (Christ and Dollinger, 2011). MSCs are able to suppress allogenic recognition responses and appear to be non-immunogenic (Ryan et al., 2005). In the human body, MSCs preferentially home to sites of injury and induce repair, either by replacing the lost cell type or by releasing growth factors and chemokines that increase the capacity of local cells to repair tissue (Prockop, 2009). Thus, human MSCs are increasingly exploited in the regeneration of damaged tissues and organs. Among others, clinical trials with MSCs are performed in the field of bone tissue engineering (Chatterjea et al., 2010), cardiac regeneration after myocardial infarction (Wollert et al., 2004), liver (Alison et al., 2000) and kidney regeneration (Poulsom et al., 2003).

The survival of cells within tissue engineered (TE) constructs, especially thicker ones, is dependent upon proper vascularization (Colton, 1995). In the hallmark paper of Folkman and Hochberg, metabolically active cells were only observed within 150-200 µm distance from their nutrient source, *i.e.* capillaries (Folkman and Hochberg, 1973). The formation of microvasculature in scaffolds is either promoted by infiltration of host cells into the scaffold

or by creating vasculature-like structures within the scaffold before implantation (Novosel et al., 2011). The latter concept is known as prevascularization and can be achieved by culturing vessel-forming cell types within the scaffolds to preform vascular structures capable of rapidly anastomosing (forming connections) with the host's blood vessels upon implantation (Laschke and Menger, 2012; Novosel et al., 2011; Rivron et al., 2008). Preformed capillary-like structures have been shown to connect with host vessels in a process called wrapping-and-tapping (WAT) anastomosis (Cheng et al., 2011). ECs within TE grafts wrap around the ingrowing host vessels, and cause degradation of the host endothelium to redirect blood flow to the engineered vascular network.

Generally, endothelial cells (ECs) or endothelial progenitor cells (EPCs) have been applied in the prevascularization approach. However, the use of mature ECs such as human umbilical vein endothelial cells (HUVECs) is associated with several disadvantages, such as the invasive harvesting procedure, their low expansion rate *in vitro* and their phenotypic heterogeneity. Thus, alternative endothelial cell sources are required to create capillary-like structures in TE scaffolds. Although embryonic stem cells (ESCs) have been successfully differentiated into endothelial cells their propensity to form teratomas *in vivo* and ethical concerns limit their clinical applicability (Hatano et al., 2013; Lees et al., 2007). Considering the plasticity of MSCs it has been proposed that these cells can be used to produce functional endothelial cells.

Several studies have confirmed the *in vitro* endothelial differentiation potential of MSCs derived from bone marrow (Janeczek Portalska et al., 2012; Liu et al., 2007), adipose tissue (Cao et al., 2005), umbilical cord (Chen et al., 2009a), and the placenta (Alviano et al., 2007; Lee et al., 2009). However, differentiation protocols varied and so did the outcome of

studies. Although bone marrow-derived MSCs acquired some phenotypic characteristics of ECs when induced in endothelial growth medium (EGM-2) for three weeks, the *in vivo* angiogenic response of these cells was not significantly improved over uninduced MSCs (Liu et al., 2007). On the other hand, TE scaffolds preseeded with MSCs that were differentiated in EGM-2 under the influence of shear stress, exhibited a significantly higher amount of ingrowing host vessels upon *in vivo* implantation than their counterparts containing HUVECs or undifferentiated MSCs (Janeczek Portalska et al., 2012; Portalska et al., 2013b).

In this study, we compared two *in vitro* endothelial differentiation protocols and evaluated the prevascularization potential of endothelial-like mesenchymal stem cells within poly(ethylene glycol) diacrylate (PEGDA) superporous hydrogels (SPHs). PEGDA SPHs contain interconnected pores ranging from 100 to 600 μm. In contrast to non-porous PEGDA hydrogel disks, acellular SPHs have been shown to promote vascular ingrowth upon transplantation into mice for four weeks (Keskar et al., 2009b). In this study, we further assessed vascularization of acellular and prevascularized PEGDA SPHs after 7 days of *in vivo* transplantation. We used the chick chorioallantoic membrane (CAM) assay to investigate the angiogenic and inflammatory response to our scaffolds.

4.2 Materials and Methods

4.2.1 Materials

PEGDA (3,400 MW) was obtained from Laysan Bio, Inc. (Arab, AL, USA). Pluronic® F127 was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). N,N,N',N'-tetramethylethylenediamine (TEMED, 99%, Acros Organics), ammonium persulfate (APS, 98+ %, Acros Organics), citric acid anhydrous, sodium bicarbonate and paraformaldehyde (96%, Acros Organics) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Chemicals were used as received without any further purification. Dulbecco's modified Eagle's medium, trypsin, penicillin and streptomycin were from Mediatech, Inc. (Cellgro®, Manassas, VA, USA). Foundation™ fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA, USA).

4.2.2   Stem cell isolation and cell culture

Stem cells were isolated from human bone marrow aspirates (AllCells, LLC; Emeryville, CA, USA) by density gradient centrifugation utilizing Ficoll-Paque™ PLUS solution followed by cell surface marker negative selection with RosetteSep® Human Mesenchymal Stem Cell Enrichment Cocktail (both from Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. HMSCs isolated from three different bone marrow donors (non-smokers, 2 males, 1 female, ages 20-31) were used up to passage four. Cells were harvested using 0.25% trypsin with 1.0 M EDTA, centrifuged, and expanded in basal medium which consists of high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 unit/mL streptomycin. Medium was changed every third day. Human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD, USA) were expanded in endothelial growth medium (EGM-2, Lonza, Walkersville, MD, USA) and used until passage four.

4.2.3   Endothelial differentiation of hMSCs

HMSCs were seeded at a density of 1,000 cells/cm^2 and incubated for 2 weeks in endothelial growth medium (EGM-2, Lonza, Walkersville, MD, USA) that contains 5 ng/mL hEGF, 0.5 ng/mL VEGF, 10 ng/mL hFGF-B, 20 ng/mL IGF-1, 1 µg/mL ascorbic acid, 0.2 µg/mL hydrocortisone, 22.5 µg/mL heparin, and 2% fetal bovine serum (FBS) (Ning et al., 2009). To one group, an additional 50 ng/mL VEGF-A (PromoCell, Heidelberg, Germany)

was added to the induction medium. We refer to this group as endothelial-like hMSCs (EMSCs) in this chapter. The controls were cultured in Dulbecco's modified Eagle's medium (DMEM)-10% FBS (basal medium).

4.2.4 Fabrication of superporous hydrogels and cell seeding

SPHs with interconnected pores ranging from 100 to 600 μm were prepared by gas foaming as previously reported (Keskar et al., 2009b; Keskar et al., 2009d; Kollmer et al., 2012). Briefly, 500 µL aqueous PEGDA solution (30% w/v), 320 µL double distilled water, 60 µL foam stabilizer (Pluronic® F-127; 10% w/v), 45 µL N,N,N',N'-tetramethylethylenediamine (TEMED; 20% w/v), 40 µL saturated citric acid solution (50% w/v) and 35 uL ammonium persulfate (APS; 20% w/v) were added sequentially to a glass vial to a final volume of 1 mL. After heating the precursor solution at 37 to 40 °C for 1 minute, sodium bicarbonate (200 mg) was stirred into the solution to evolve CO_2 by reacting with the citric acid. Polymerization and gas formation occurred simultaneously to create an interconnected porous network. After 30 min, SPHs were removed from the vial and washed three times in double-deionized water to remove traces of unpolymerized monomers and salt before sterilizing the gels in 80% ethanol overnight, followed by dehydration in absolute ethanol for 4 hours. SPHs were dried in a food desiccator at a temperature of 55°C for approximately 2 hours. Dried hydrogels were cut to give scaffolds with a diameter of 5 mm and a thickness of 1 mm. Cells (5×10^5) were resuspended in rat tail collagen type I (BD Bioscience, Bedford, MA, USA) to create a 3% gel. Cell-collagen solution (50 uL) was added dropwise to the SPH scaffold. Three groups of cell-seeded constructs were prepared and cultured for 14 days in the respective medium: i) HUVECs-containing SPH-Col constructs were maintained in EGM-2 medium, ii) hMSCs-containing SPH-Col constructs

were maintained in basal medium, and iii) hMSCs were induced towards EMSCs within SPH-Col constructs with EGM-2 and VEGF (50 ng/mL).

4.2.5 Flow cytometry

After 7 and 14 days of endothelial induction, flow cytometric analysis was conducted to monitor the expression of endothelial markers. Cells were washed in 1X DPBS, trypsinized (0.25% trypsin, 2.21 mM EDTA) and washed in FACS buffer (1X DPBS + 5% FBS + 0.05% 3M sodium azide). After centrifugation, cells were incubated on ice for 30 min in 100 µL of FACS buffer containing anti-human CD309-AF647 and CD34-PE. Cells were washed twice with FACS buffer and finally diluted into 100 µL of FACS buffer. Fluorochrome- and isotype-matched antibodies were used as controls. All antibodies were obtained from Biolegend (San Diego, CA, USA) and used at manufacturer's recommended concentrations. Analysis was performed by collecting 15, 000 events on a Beckman Coulter Cyan ADP.

4.2.6 Matrigel assay

At day 14 of endothelial differentiation, the Matrigel assay was applied to investigate the network formation ability of the induced hMSCs. Uninduced hMSCs served as control. Matrigel (BD Biosciences, Bedford, MA, USA) was added to a 48-well plate (80 µL/ well) and allowed to solidify for 30 min at 37 ºC. Cells (1.5×10^4) that were cultured for 14 days in EGM-2 alone or with additional VEGF (50 ng/mL) and uninduced cells were suspended in basal medium, EGM-2 and EGM-2 plus VEGF and placed on top of the matrigel. After 24 hours incubation, the formation of a tube-like network was examined by taking images on an Olympus IX70 inverted microscope.

4.2.7 Cell viability within SPHs

For live-dead staining, wells containing the cell-seeded constructs were rinsed with 1X DPBS to remove serum and medium components. A solution comprising 4 mM calcein acetoxymethyl ester and 4mM ethidium homodimer in 1X DPBS was added to the SPHs and allowed to incubate for 25 min at 37°C. SPHs were rinsed twice with 1X DPBS and fluorescent images were taken with an Olympus IX70 inverted microscope.

4.2.8 Chick chorioallantoic membrane (CAM) assay

Fertilized eggs from a local farm (Sunnyside, Inc) were incubated horizontally at 37°C for 7 days in an Octagon® 20 Eco incubator. On day 7, a small hole was created in the tip of the egg (where the air bubble resides) and a second hole was formed in the elongated side of the egg (where the air bubble should be moved) with the aid of a small dissecting scissor. By squeezing a rubber bulb over the second hole, we created suction and moved the air bubble from the tip to the elongated side. Then, an approximate 1 cm x 1 cm window was cut into the shell at the side of the air bubble (creates space between the CAM-embedded embryo and the eggshell), the window was sealed with tape and the eggs were put back into the incubator. On the following day, embryos were monitored for survival. Scaffolds were only applied to CAMs of live embryos. Five experimental groups were tested: SPH scaffolds without collagen and cells (SPH), SPH composite with collagen embedded into the pores of the scaffolds (SPH-Col), SPH scaffolds that were precultured with collagen-embedded HUVECs (SPH-Col-HUVECs), SPH scaffolds that were precultured with collagen-embedded hMSCs (SPH-Col-hMSCs), and SPH scaffolds that were precultured with collagen-embedded EMSCs (SPH-Col-EMSCs). After the grafting procedure, the window was resealed and the eggs were further incubated for another week. On day 15, scaffolds and the connected CAM

were cut out using forceps and dissecting scissor, fixed in 4% paraformaldehyde, dehydrated in an ethanol series, embedded in paraffin and sectioned for histological analysis.

4.2.9   Blood vessel count

Hematoxylin & eosin (H&E) sections of the retrieved CAM were used to count blood vessels in scaffold vicinity. Images of the CAM were randomly taken in areas that were adjacent to the scaffold. Blood vessels were counted in 6 areas per H&E stained CAM section with 3 sections per group.

4.2.10 Immunofluorescence

Histological sections of the retrieved CAM and the scaffolds were stained with mouse anti-chicken CD34 antibody (AbD Serotec) to visualize the infiltration of endothelial cells into the scaffolds. Deparaffinized and antigen-retrieved slides were treated with blocking solution (1% BSA in 1X PBS). After 30 min of incubation, the CD34 antibody was added to the blocking solution and slides were further incubated overnight at 4°C. Slides were washed with 1X PBS and incubated with AlexaFluor®488-labeled goat anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA) for 1 hr at room temperature (RT). The nucleus was counterstained with H33258 (1 mg/mL; Molecular Probes, Carlsbad, CA) for 5 min at RT. Images were taken with an Olympus IX70 inverted microscope and processed using QCapture Pro software.

4.3 Results

4.3.1   Endothelial marker expression and morphology of induced MSCs

Endothelial growth medium 2 (EGM-2) with or without additional VEGF-A has been used in several previous studies to induce endothelial differentiation of human mesenchymal stem

cells (hMSCs) (Lee et al., 2009; Liu et al., 2007). To investigate the effect of VEGF addition, we cultured MSCs in EGM-2 alone or in EGM-2 with additional 50 ng/mL VEGF-A. Uninduced controls were maintained in basal medium (DMEM-10% FBS) for the entire culture period of 14 days. HUVECs served as vascular endothelial cell control for the endothelial marker expression.

To get an initial idea about the endothelial differentiation potential of hMSCs and to assess the tested culture media, we monitored the expression of endothelial markers, CD309 and CD34, with flow cytometry after 7 and 14 days of differentiation. CD309, a transmembrane tyrosine kinase receptor, also known as vascular endothelial growth factor receptor-2 (VEGFR-2) or kinase insert domain–containing receptor (KDR). CD34, a type I membrane glycophosphoprotein, is expressed on small vessel endothelial cells and hematopoietic progenitor cells.

We did not detect any expression of endothelial markers in MSCs cultured under basal conditions or in EGM-2 alone for the culture period of 14 days. After 14 days of induction, we observed a significant increase in CD309 expression in the VEGF supplemented group but no significant change in CD34 expression was observed. Our results indicate that endothelial marker expression is dependent upon both medium composition and differentiation time (**Figure 4.1a)**. It is suggested that VEGF plays an important role in the endothelial differentiation process of MSCs. Although cells that were induced with VEGF supplemented EGM-2 acquired a more compact morphology, we did not observe the cobblestone-like morphology typical for endothelial cells (**Figure 4.1b)**. Uninduced MSCs exhibited a fibroblast-like, spindle shaped morphology and EGM-2 treated MSCs appeared to be more elongated compared to the other groups. Based on this data it is still unclear

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whether "true" endothelial cells can be obtained with the current differentiation protocols. Thus, we tested the functional features of the endothelial-like hMSCs on matrigel.

4.3.2 Tube formation capacity of induced MSCs

We compared the angiogenic capacity of undifferentiated cells and of cells that were pre-differentiated in EGM-2 with or without additional VEGF for 14 days using the *in vitro* tube formation assay on matrigel. Capillary-like structures could be observed to a similar extent in the induced cells as well in the uninduced cells when the tube formation process was conducted in endothelial growth medium with or without additional VEGF for 24 hours on matrigel (**Figure 4.2**). Interestingly, only cells that were maintained in EGM-2 medium with additional VEGF for 14 days formed tubes when cultured in basal medium on matrigel for 24 hours. The data indicate that cells that were pre-differentiated in endothelial growth medium with additional VEGF act more like endothelial cells on matrigel even in conditions where no growth factors are present, *i.e.* in basal medium. Those results are in accord with the observed endothelial marker expression. Only cells that were differentiated in EGM-2 with additional VEGF stimulated a significant upregulation of the endothelial marker CD309. Thus, we found that bone marrow-derived hMSCs acquire phenotypic and functional characteristics of ECs and further tested the potential of these cells in a TE application. In all following experiments in this study, we refer to cells that were induced with VEGF supplemented EGM-2 for 14 days as endothelial-like hMSCs (EMSCs).

4.3.3 Cell viability and capillary network formation within SPHs

The majority of cells within the SPH-Col constructs remained viable after 14 days of *in vitro* culture (**Figure 4.3**). Some dead cells were observed in HUVEC- and EMSC-containing constructs. No dead cells were seen in hMSC-containing scaffolds. Tube-like

structures were present in scaffolds that contained differentiated hMSCs (EMSCs) but not in HUVEC- or hMSC-containing scaffolds. HUVECs have been shown to align into vascular networks on collagen gel; however these structures tend to be thin and immature (Nakatsu et al., 2003). Although we observed some dead cells within the EMSC-containing constructs, the cells were still able to maintain tube-like structures over a period of 14 days. It is suggested that while forming vascular structures, EMSCs play the role of endothelial cells that form vessels as well as the role of stabilizing pericytes (da Silva Meirelles et al., 2008; Janeczek Portalska et al., 2012).

4.3.4 *In vivo* response of scaffolds after 7 days on CAM

Angiogenic response

Histological sections of the retrieved scaffolds were stained with hematoxylin & eosin (H&E) and Masson's trichrome staining to monitor infiltration of tissue and vessels. H&E stains nuclei dark purple to black and stains cell cytoplasm pink (Kiernan, 2008). Masson's trichrome stains nuclei black, cytoplasm red and collagen blue (Kiernan, 2008). In each treatment group we observed host cells infiltrating the scaffold (**Figure 4.5, 4.7**). The depth of tissue invasion was similar in each treatment group and cells in the middle of the scaffold could be observed in each group. Luminal structures and avian CD34 positive cells indicating blood vessel ingrowth were observed in all groups (**Figure 4.5, 4.7**). Some of those lumen contained bright red avian blood cells (**Figure 4.5**). Vessels containing remodeled collagen were observed in all scaffolds that were precultured with cells but were most prominent in scaffolds containing HUVECs and EMSCs (**Figure 4.6**). The angiogenic potential of the scaffolds was evaluated by the presence of microvessels in close proximity to the CAM/scaffold interface in the CAM stroma. Cell- and collagen-free SPHs and EMSCs-

containing scaffolds appeared to promote the highest blood vessel density in the CAMs whereas CAMs close HUVEC-containing scaffolds appeared to have the lowest vessel density but these differences were not statistically significant (**Figure 4.8, 4.9**).

Inflammatory response

The CAM is circa 200 μm thick and consists of a multilayer epithelium at the air interface (ectoderm), a loose stroma, and a one-cell layer-thick inner epithelium (endoderm) (Barnhill and Ryan, 1983). All scaffolds were integrated in the CAM but did not get completely absorbed (**Figure 4.4**). We did not observe any giant cells of the foreign body type in any of the groups indicating the absence of a mature foreign body reaction. However, a mild inflammatory response was observed as indicated by the increase in thickness of the stroma and the development of dense tissue in this layer. We further detected squamous metaplasia of the ectoderm at the CAM/scaffold interface (**Figure 4.7**). In CAMs next to SPH-Col-HUVECs and SPH-Col-hMSCs, the ectoderm layer had disappeared upon contact with the scaffolds (**Figure 4.7**). Then again, trichrome staining revealed the absence of collagen infiltration in cell-free SPH constructs indicating the absence of granulation tissue formation (**Figure 4.6**). SPH-Col, SPH-Col-hMSCs, SPH-Col-HUVECs and SPH-Col-EMSCs constructs contained few areas of collagen that seemed to be part of vascular structures. The observed responses indicate a high biocompatibility of the tested constructs.

The collagen type I solution which was embedded in the SPH was still detectable after 7 days of implantation onto the CAM but was not stained blue with the aniline dye of the trichrome stain. The interaction of proteins of a tissue and a fixative agent leads to the formation of a three-dimensional, insoluble protein network. Structure and density of these networks may influence the staining results of the respective tissue. Collagen networks

exhibit an open pore structure and allow larger dyes like aniline blue to penetrate. SPHs were immersed with collagen type I that forms a triple helix network composed of two α1 chains and one α2 chain at neutral pH and 37 °C. A denser collagen network might have formed with the collagen type I solution than with the collagen secreted by avian cells and thus, the aniline dye was not able to penetrate.

4.4 Discussion

Proper vascularization is not only crucial for the success of cell-seeded scaffolds after transplantation it also important for the *in vitro* generation of engineered tissues (Rivron et al., 2008). The presence of ECs in TE constructs has been shown to affect the performance of coseeded cell types. For instance, the interplay between HUVECs and hMSCs in a spheroid co-culture model was responsible for the upregulation of osteogenic markers suggesting an improved differentiation of the osteoprogenitor cells (Rouwkema et al., 2006). The objective of this investigation was (a) to establish an *in vitro* endothelial differentiation protocol for bone marrow-derived hMSCs, and (b) to evaluate if preseeding of scaffolds with endothelial-like hMSCs promotes the *in vivo* angiogenic response.

After 14 days of induction in EGM-2 with additional VEGF (50 ng/mL), we observed a significant increase in CD309 but not in CD34 expression. When hMSCs were cultured in stem cell maintenance medium or in EGM-2 alone, no endothelial marker was upregulated suggesting an important role of VEGF in the endothelial differentiation process of hMSCs (**Figure 4.1a**). VEGF is known to play a major role in the initiation of angiogenesis and is involved in the activation of pericellular proteolysis, the increase of vascular permeability, and the stimulation of EC proliferation and migration with subsequent lumen formation (Breuss and Uhrin, 2012; Nomi et al., 2006). A recent study suggests that VEGF induces

differentiation of human and rat bone marrow-derived MSCs to ECs by Rho/ROCK signaling-mediated nuclear translocation of myocardin-related transcription factor-A (MRTF-A) (Wang et al., 2013b). CD309, also referred to as VEGF-R2 or KDR, is mainly expressed in vascular endothelial cells where it acts as the principal receptor transmitting VEGF signals (Hoeben et al., 2004; Holmes et al., 2007). The induction of CD309 expression upon stimulation of MSCs with endothelial growth medium containing at least 50 ng/mL VEGF-A was observed in bone-marrow- (Oswald et al., 2004), umbilical cord- (Chen et al., 2009a) and placenta-derived MSCs (Lee et al., 2009).

Similar to our observation, CD34 expression was negative in bone marrow-derived MSCs when induced in VEGF-containing medium for 7 days (Oswald et al., 2004). However, when induced with identical amounts of VEGF, placenta-derived MSCs started to express CD34 after 7 days of differentiation suggesting variations in the endothelial differentiation potential between different tissues of origin (Alviano et al., 2007). To evaluate the functional behavior of the differentiated cells, we tested their network formation ability on Matrigel (**Figure 4.2**). The ability to form capillary-like structures was similar in uninduced and induced hMSCs when the tube formation process was conducted in endothelial growth medium. However, only cells that were induced in VEGF-supplemented EGM-2 medium, formed tubes when cultured in basal medium on Matrigel for 24 hours (**Figure 4.2**). Thus, preconditioning with VEGF promotes the formation of cells that act more like endothelial cells on Matrigel and form tubes even under less promoting conditions (basal medium). We showed that hMSCs acquire some phenotypic and functional features of endothelial cells when cultured in EGM-2 plus additional VEGF (50 ng/mL) for 14 days. We refer to these cells as endothelial-like cells (EMSCs) in this study.

We also induced endothelial differentiation of hMSCs directly within SPH-Col gels and evaluated the *in vitro* and *in vivo* angiogenic response of these constructs. After 14 days of endothelial induction, capillary-like structures were observed within constructs containing the induced hMSCs (SPH-Col-EMSC group) while no vascular structures were seen in HUVEC- and uninduced hMSC-containing SPH-Col scaffolds (**Figure 4.3**). Recent studies showed that overexpression of the caspase-resistant Bcl-2 protein (Schechner et al., 2000) or co-culture with fibroblasts (Chen et al., 2009b) was necessary to create mature microvessel networks with HUVECs *in vitro*. Our results are in agreement with previous studies and show the benefit of EMSCs (over HUVECs) for *in vitro* prevascularization approaches.

Up to now only a few studies examined the applicability of endothelial-like MSCs in TE settings (Janeczek Portalska et al., 2012; Portalska et al., 2013a; Portalska et al., 2013b; Sahar et al., 2012). Preseeding of a porous scaffold with Matrigel-embedded endothelial-like MSCs led to a greater angiogenic response *in vivo* than prevascularization with MSCs or HUVECs (Janeczek Portalska et al., 2012). While, *in vivo* vascularization and bone formation of poly (D,L-lactide) (PLA) scaffolds that were seeded with endothelial-like MSCs, MSCs that underwent osteogenic differentiation or undifferentiated MSCs prior to implantation into mice for 8 weeks (Sahar et al., 2012). Scaffolds containing endothelial-like MSCs exhibited a higher (although not statistically significant) microvessel density but bone formation was significantly reduced compared to other cell-seeded constructs. Another study demonstrated that scaffolds containing co-cultures of MSC-derived ECs and MSCs displayed a higher amount of regenerated bone compared to MSC-seeded scaffolds when implanted into bony defects of the rabbit mandible (Liu et al., 2013). Thus, there is evidence of the

potential of endothelial-like MSCs in supporting vascularization and bone formation within TE scaffolds. Thus, we further tested our prevascularized constructs *in vivo.*

As a first assessment of in vivo vascularization, we used the chick chorioallantoic membrane (CAM) assay to evaluate angiogenic and inflammatory responses of the cell-seeded scaffolds (Spanel-Borowski, 1989). The CAM is an extra-embryonic membrane that provides a gas exchange surface to the chicken embryo during the 21 days of development (Ribatti et al., 2006). Immature vessels begin to form on day 4 of incubation by fusion of the allantois (ectodermal epithelium) with the chorion (endodermal epithelium) to form the chorioallantois. At day 8, vessels differentiate into capillaries and continue to grow rapidly until incubation day 12 where the CAM reaches its maximum vascular density. Scaffolds can be grafted on top of the membrane on days 7 to 9 by creating a window in the eggshell. Vessels grow perpendicularly to the plane of the CAM inside the scaffold. After the implantation period (no longer than 10 days since chicken hatch on day 21) the grafts can be excised and processed for histology. The assay does not require an animal facility or protocol, and is thus relatively inexpensive compared to other *in vivo* models. However, high mortality rates of the chicken embryos and a short study timespan are limitations of the CAM method.

We implanted our TE constructs for 7 days onto the CAM. Blood vessel infiltration was observed in all groups but mature, collagen-surrounded blood vessels were only present in SPHs that were prevascularized with EMSCs or the standard endothelial cell line (HUVECs) (**Figure 4.5-4.7**). Cell- and collagen-free SPHs and SPH-Col-EMSC constructs showed the highest angiogenic response and SPH-Col-HUVEC scaffolds the lowest (**Figure 4.8 and 4.9**). However, these differences were not statistically significant. We believe that an increase

in samples per group (currently N=3) will resolve this issue. Interestingly, the ingrowth of avian CD34 positive endothelial cells was most prevalent in cell- and collagen-free SPHs suggesting that the porous architecture of the scaffold *per se* is a driving force in the revascularization process of these scaffolds *in vivo* (**Figure 4.7**). However, mature collagen-surrounded vascular structures were only present in cell-containing constructs and most prominent in the SPH-Col-EMSC and SPH-Col-HUVEC scaffolds indicating the potential of the prevascularization approach. Further investigations are warranted to explain why SPH-Col-HUVEC scaffolds promote the ingrowth of mature blood vessels but exhibit an overall lower angiogenic response.

In the CAM assay, tissue reactions to biomaterials such as acute and chronic inflammation, formation of granulation tissue, and fibrosis are similar to that of mammals (Klasing, 1991; Valdes et al., 2002). Despite the many advantages, the CAM assay is rarely used today. In 1989, Spanel-Borowski used the CAM assay to test several biomaterials used for vascular prosthesis and temporary skin substitutes (Spanel-Borowski, 1989). In this study, inflammatory cells such as heterophils and giant cells were found more often in gelatin sponges than in collagen sponges. It was also observed that synthetic materials such as expanded polytetrafluorethylene (ePTFE) induced squamous metaplasia of the chorion epithelium and Dacron and polyurethane foams even induced ulceration of the CAM. Interestingly, *in vivo* healing rates of ePTFE appear to be lower than those of Dacron prostheses due to the limited ingrowth of fibroblasts and granulation tissue (Spanel-Borowski, 1989).

Three of our tested constructs contained human cells but the lack of a mature immune system during embryonic chick development permits the use cells from any species without

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inducing significant immunologic responses (Boulland et al., 2010). In the early phases of chick development eosinophilic granulocytes and messengers of a histocompatibility complex are lacking (Spanel-Borowski, 1989). The CAM method also serves as a model system in cancer research to visualize tumor angiogenesis and metastasis upon application of cancer cells or tumors (Deryugina and Quigley, 2008). After 7 days of implantation onto the CAM, none our tested constructs induced a mature foreign body reaction (**Figure 4.7**). Only mild inflammatory responses were detected such as the increase in thickness of the stroma and squamous metaplasia of the ectoderm at the CAM/scaffold interface (**Figure 4.7**). The absence of substantial amounts of collagen-containing granulation tissue within our constructs is in accord with a previous published study. Macroporous PEG hydrogels started forming significant amounts of granulation tissue at week 2 and 3 post implantation into rats (Chiu et al., 2011) which would be too late to be observed in this study. The introduction of cells within the PEGDA SPHs increased the formation of collagen-containing vascularized tissue within the scaffold pores suggesting a benefit of this approach.

4.5 Conclusion

Recent advances in tissue engineering suggest that forming vascular structures within scaffold materials *in vitro* improves the vascular response after implantation. Thus, cells that can be expanded in large numbers and possess vessel-forming capabilities are desired. We found that bone marrow-derived hMSCs acquire several endothelial-like characteristics when cultured in endothelial growth medium that was supplemented with VEGF (50 ng/mL). Endothelial-like hMSCs contributed to the formation of capillary-like structures within PEGDA superporous hydrogels. When implanted onto the chick chorioallantoic membrane for 7 days, the prevascularized scaffolds contributed to the ingrowth of mature, collagen-

surrounded blood vessels in a similar way than scaffolds that were preseeded with HUVECs, the standard endothelial cell line. Cell-free and prevascularized scaffolds exhibited a mild inflammatory response but no foreign body reaction was observed. Our data suggest a potential benefit of MSC-derived ECs in the prevascularization approach but further studies are warranted to elucidate the stability and functionality of the *in vitro* formed capillary-like structures.
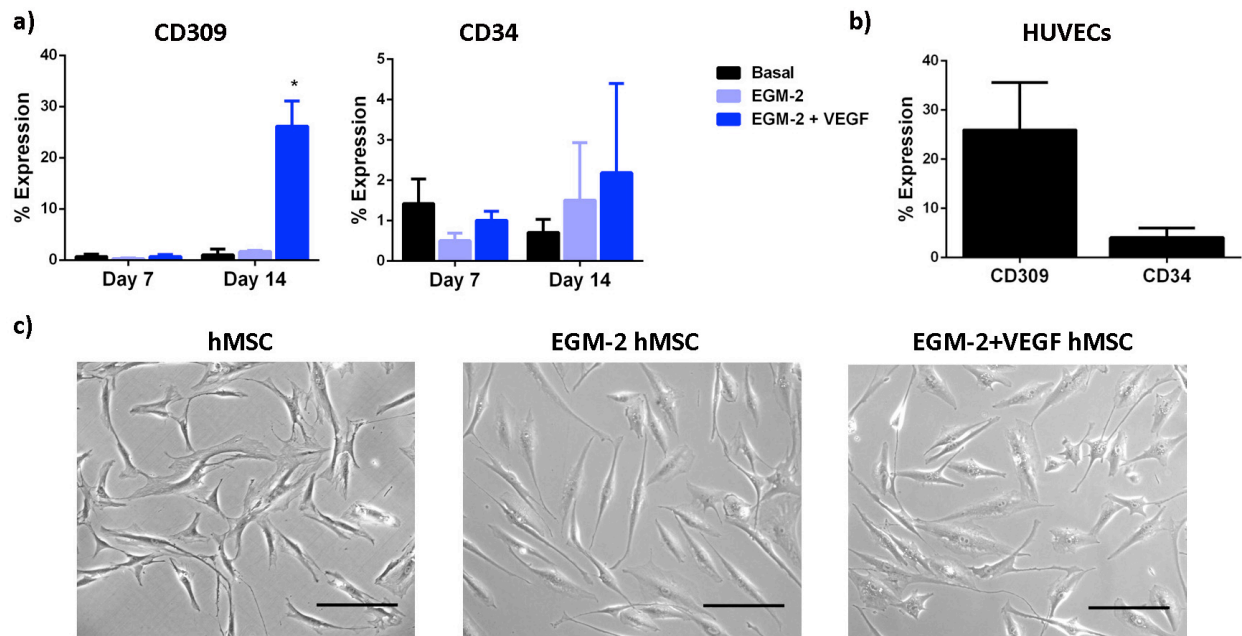
Figure 4.1 Evaluation of the endothelial phenotype of differentiated hMSCs (EMSCs). (a) Flow cytometry results of endothelial marker expression CD309 (VEGF-R2) and CD34 in hMSCs that were cultured for 14 days in basal, EGM-2 or EGM-2 + 50 ng/µL VEGF-A medium. The symbol (*) denotes a significantly higher CD309 expression in the VEGF-supplemented EGM-2 medium at day 14 compared to all other groups (n=3; mean ± standard deviation). (b) Marker expression of tissue culture plastic expanded HUVECs served as positive control (n=3; mean ± standard deviation). (c) Morphology of hMSCs expanded in basal, EGM-2 or EGM-2 + 50 ng/µL VEGF-A medium for 14 days. The latter ones are referred to as endothelial-like MSCs (EMSCs). Scale bar is 100 µm.
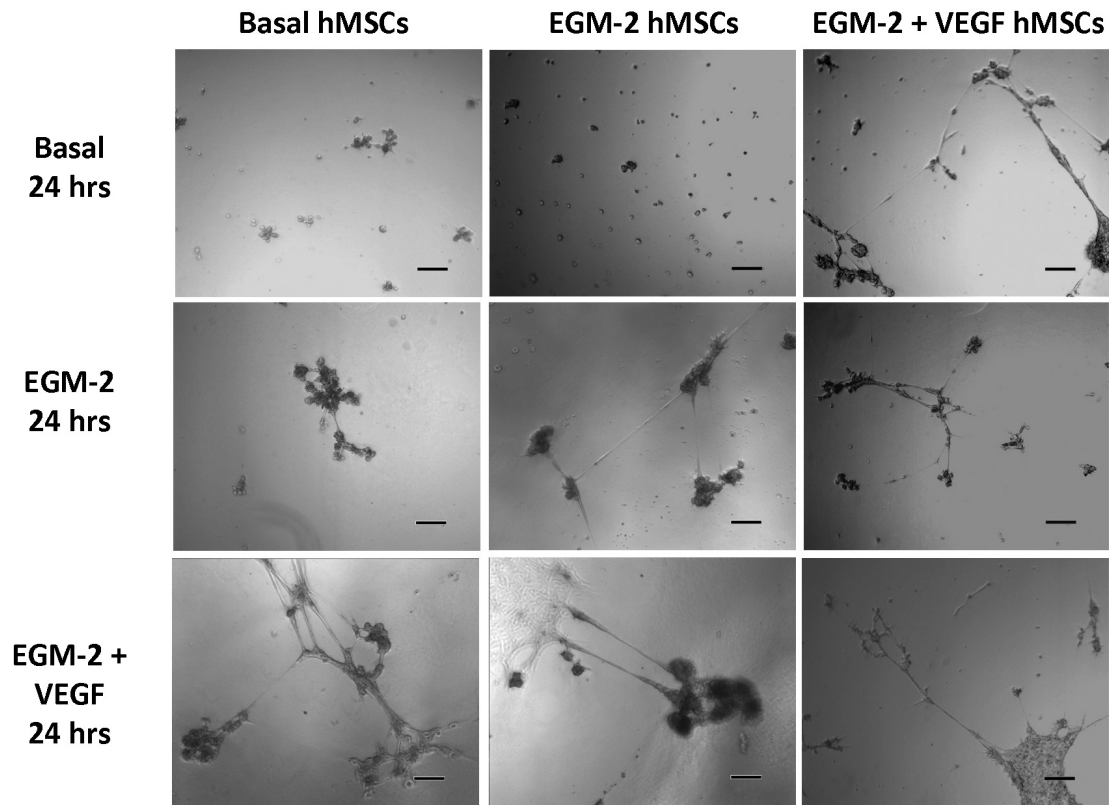
Figure 4.2 Formation of capillary-like structures on Matrigel. HMSCs were cultured for 14 days in basal (left column), EGM-2 (middle column) or EGM-2 with an additional 50 ng/mL VEGF-A (right column) and then seeded on matrigel and cultured in basal (upper row), EGM-2 (middle row) or EGM-2 with an additional 50 ng/mL VEGF-A (lower row) for 24 hours. Scale bar is 100 μm.
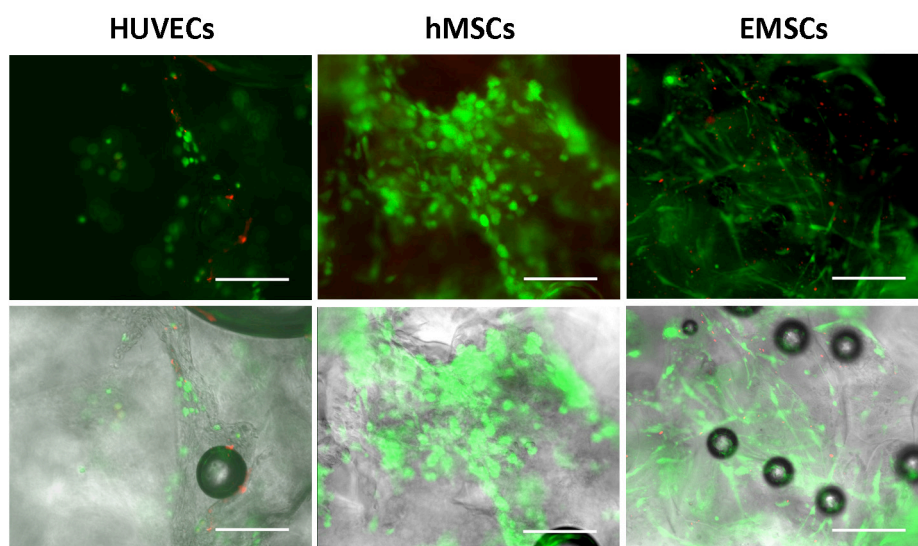
Figure 4.3 Live and dead staining after 14 days of *in vitro* prevascularization. Representative live (green)-dead (red) pseudocolor stained images of scaffolds that were cultured with HUVECs, MSCs or EMSCs are presented. The upper row shows fluorescence pictures and the lower row is an overlay of the fluorescent pictures and a brightfield picture of the scaffold. The scale bar is 100 μm.
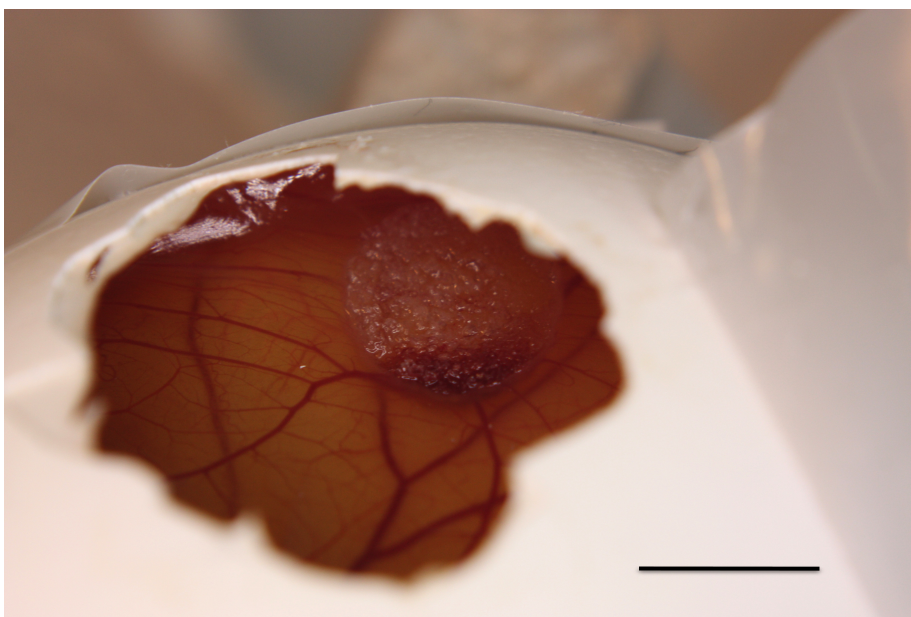
Figure 4.4 Representative photograph of SPH scaffold after 7 days on the CAM. The scale bar is 1 cm.
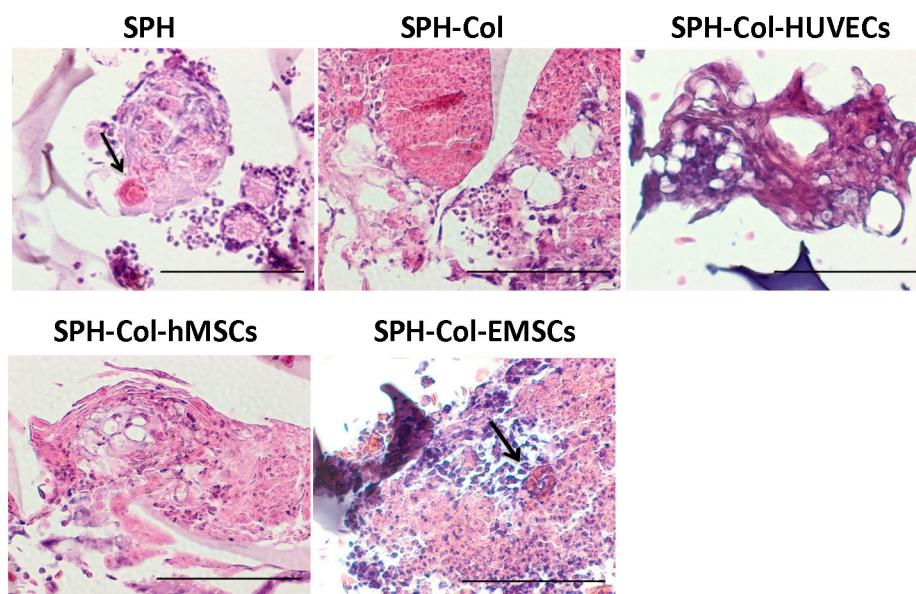
Figure 4.5 H&E stained hydrogels after 7 days on the CAM. Representative pictures of SPH scaffolds without collagen and cells (SPH), collagen-loaded SPH scaffolds (SPH-Col), SPH scaffolds that were precultured with collagen-embedded HUVECs (SPH-Col-HUVECs), SPH scaffolds that were precultured with collagen-embedded hMSCs (SPH-Col-hMSCs), and SPH scaffolds that were precultured with collagen-embedded EMSCs (SPH-Col-EMSCs). Luminal structures indicating vascular infiltration were observed in all groups. Some of those lumen contained bright red avian blood cells. The black arrows point at blood perfused vessels. The scale bar is 100 μm.

SPH **SPH-Col** **SPH-Col-HUVECs**

SPH-Col-hMSCs **SPH-Col-EMSCs**

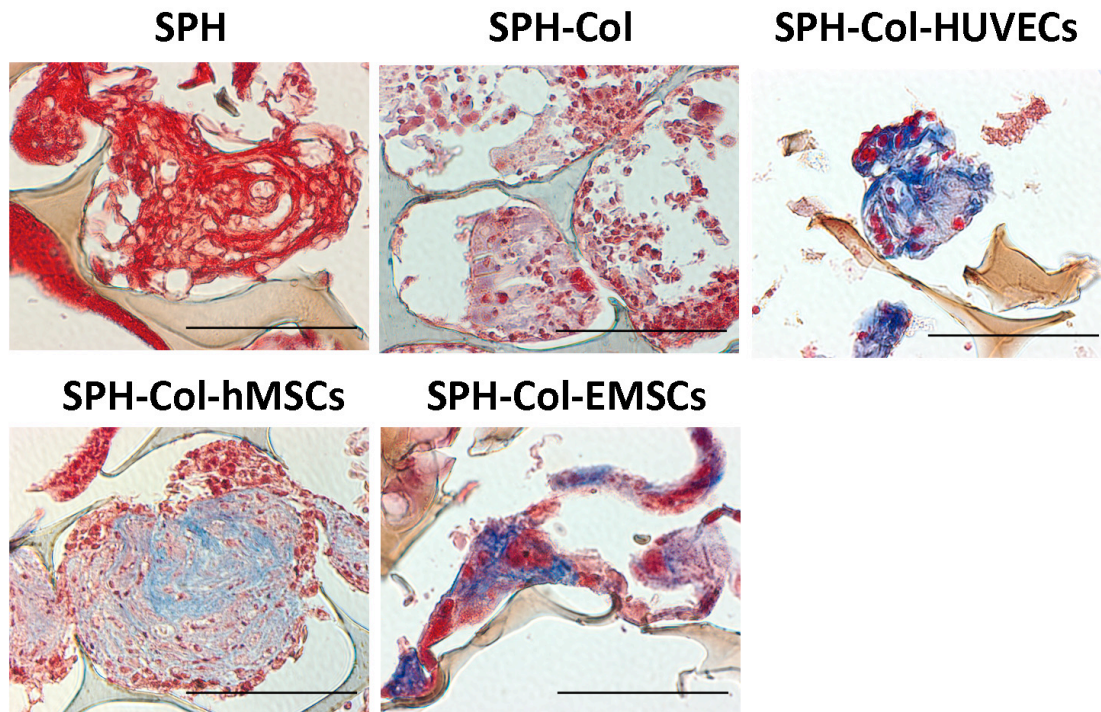**Figure 4.6 Masson's trichrome stained gels after 7 days on the CAM.** No collagen (blue stain) was observed in SPHs without collagen gel and cells. Some collagen-infiltrated areas were observed in the SPH-Col and SPH-Col-hMSCs group. Scaffolds containing HUVECs and EMSCs contained blood cells that are surrounded by collagen. The scale bar is 100 μm.
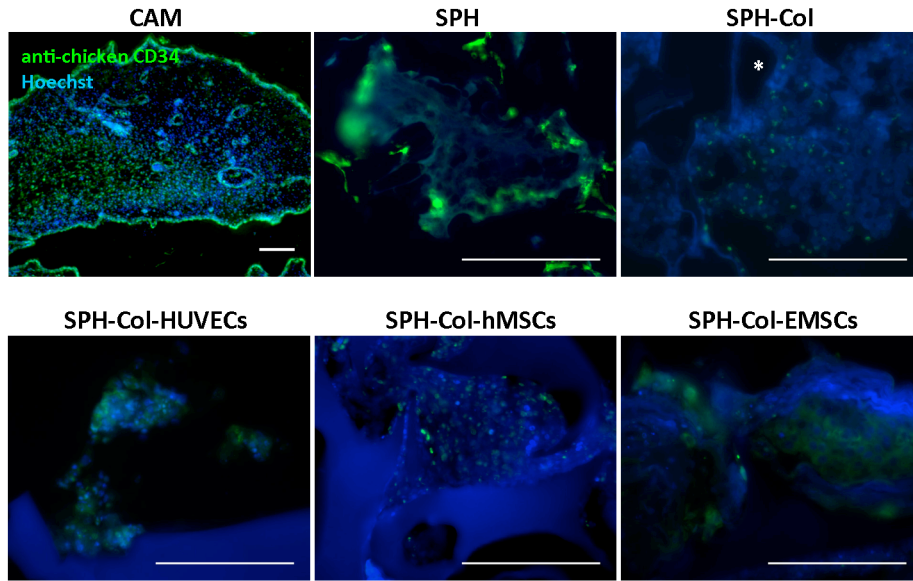
Figure 4.7 Avian CD34+ endothelial cells infiltrated the scaffolds. Representative immunofluorescent images of CD34 (green) expressed by infiltrating avian endothelial cells into the scaffolds without collagen and cells (SPH), collagen-loaded SPH scaffolds (SPH-Col), SPH scaffolds that were precultured with collagen-embedded HUVECs (SPH-Col-HUVECs), SPH scaffolds that were precultured with collagen-embedded hMSCs (SPH-Col-hMSCs), and SPH scaffolds that were precultured with collagen-embedded EMSCs (SPH-Col-EMSCs) are shown. The scaffolds were implanted onto the CAM for 7 days. The scale bar is 100 μm.

CAM **SPH** **SPH-Col**

SPH-Col-HUVECs **SPH-Col-hMSCs** **SPH-Col-EMSCs**
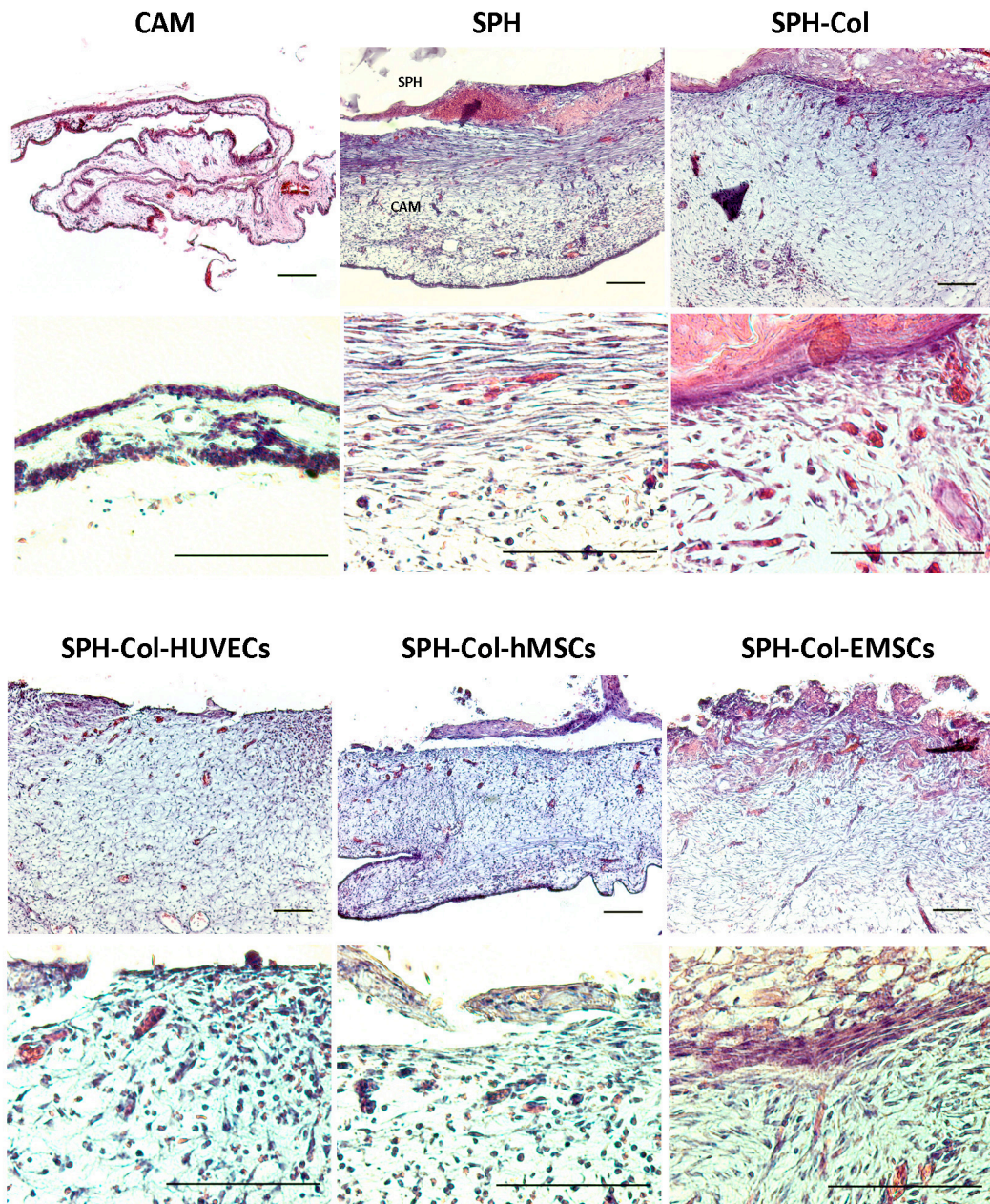
Figure 7.8 Light micrographs of H&E stained CAM sections and adjacent scaffolds after 7 days of implantation. The scaffolds were not embedded in a collagen capsule and no giant body cells were observed indicating the absence of a mature foreign body reaction. The scale bar is 100 µm.
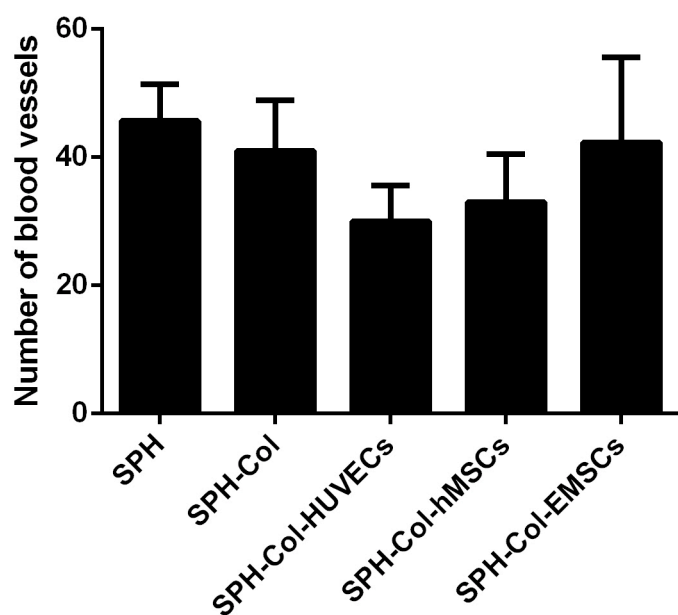
Figure 4.9 Quantification of CAM blood vessels in scaffold vicinity. The mean number of blood vessels results from the counts of 6 areas (40x magnification images in areas adjacent to the scaffold) of H&E stained CAM section with 3 sections per group.

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5 General conclusions and future directions

5.1 A tissue engineering standpoint

Tissue engineering (TE) translates fundamental knowledge in biology, chemistry, and physics into materials, devices, and strategies to induce regeneration or repair of human cells, tissues or organs (Muschler et al., 2004). Commercially available TE products include engineered human skin equivalents (Transcyte™, Apligraf®, Dermagraft®), heart valve replacements (Cryovalve®SG pulmonary heart valve), as well as cartilage (Hyalograft®C) and bone substitutes (Regenafil®, Vitoss®Scaffold FOAM™) for human use. As of this writing, there are no FDA approved stem cell-based tissue engineered products on the market yet. However, numerous experimental studies and initial clinical observations underwrite the potential of stem cell-based TE.

The implementation of adult stem cells has increased the options for TE. Stem cells can be differentiated towards tissue-forming cells and can be used to recreate tissues *in vitro*. Since cells cannot form intricate tissues by themselves, a template or a scaffold is needed to provide control over tissue architecture. Thus, TE transitioned from a historically materials-based to a cell-based or bioactive materials approach. Cell-based TE focuses on cell function and the role of materials, scaffolds and other bioactive stimuli in modulating cell behavior. Yet, considerable challenges remain before the clinical goals of stem cell-based TE constructs can be achieved.

One challenge is the design of scaffold materials that are able to keep stem cells in their undifferentiated functional phenotype and promote differentiation only after induction. The maintenance of stem cells in their undifferentiated state allows for site-specific cell responses, and thus could be advantageous in many TE applications where optimal tissue

integration is required. For tissue formation, higher cell numbers are required and scaffolds that promote cell proliferation and survival are required. Another challenge in stem cell-based TE is the identification of reliable stem cell differentiation markers.

The expression of putative differentiation markers often varies in *in vitro* differentiation protocols due to donor variability. However, in the process of bone regeneration, it was recently shown that the large variability of *in vitro* differentiation capability did not correlate with *in vivo* ectopic bone formation (Mentink et al., 2013). A single gene, CADM1, was strongly linked to the bone-forming capacity of hMSCs and was suggested as an *in vitro* diagnostic marker. Further, most studies do not exclude the potential for expression of alleged marker genes in other differentiated lineages. Thus, for stem-cell based TE to advance into the clinic, reliable marker genes indicating differentiation towards a certain tissue-specific lineage, ruling out differentiation towards another lineage and eventually predicting the *in vivo* performance are essential.

Another reason for the limited clinical success with current cell-based TE grafts is the inability to effectively vascularize tissues *in vitro* and *in vivo*. Insufficient oxygen and nutrient supply within the core of the implants restricts the survival of engrafted cells after implantation. Recent advances in TE suggest that *in vitro* preformed vascular structures improve the vascular response after implantation. Thus, cells with vessel forming capabilities that can be expanded in large numbers are required.

This dissertation research was specifically designed 1) to evaluate the potential of PEGDA superporous hydrogels (SPHs) as TE scaffolds suitable for the delivery of viable and functional stem cells, 2) to assess commonly used adipogenic and osteogenic marker genes for their specificity towards the respective lineage, and 3) to assess the *in vitro* and *in vivo*

vascularization potential of endothelial-like mesenchymal stem cells within SPHs. The outcome of these studies has not only answered specific questions but also opened the way for new studies.

5.2 Conclusions and future directions

The first part of this research project was aimed to understand how physicochemical characteristics of a TE scaffold impart properties that could foster cellular responses (chapter 2). HMSCs do not survive on top of unmodified, nonporous PEGDA hydrogels. However, when seeded within the porous network of PEGDA SPHs we observed viable cells for over seven weeks. We found that the initial attachment of hMSCs to PEGDA SPHs occurs via serum proteins that have been adsorbed onto the scaffold surface. However, this effect was not observed when the hMSCs were cultured on the nonporous hydrogels (Keskar et al., 2009d). Interestingly, the presence of serum proteins is required for initial anchorage of hMSCs within the SPHs but not for hMSC survival after 24 hours. When the culture medium was switched to serum-free medium after 24 hours, hMSC viability within SPHs was similar to their counterparts that were cultured in serum-containing medium for 3 weeks. Thus, after initial attachment to the scaffolds, PEGDA SPHs provide an option for serum-free culture. For translating tissue engineered constructs into the clinic, it is especially important to design culture conditions without animal products to reduce the risk of infection or contamination.

We are the first ones that showed the presence and the development of cell-matrix interactions within unmodified macroporous PEGDA hydrogels. We found that hMSCs deposit their own extracellular matrix (ECM) within SPHs and detected the expression of collagen type I, collagen type IV, fibronectin and laminin on mRNA and protein level within hMSC-seeded PEGDA SPHs. We believe that the cell-secreted ECM supports long-term

survival of hMSCs within the PEGDA SPHs by promoting cell attachment via integrin-mediated signaling and further aids in creating a niche environment. In the human body, the ECM regulates, together with neighboring differentiated cells, the balance between stem cell maintenance and differentiation. After long-term culture within PEGDA SPHs, hMSCs expressed stem cell surface markers, CD105, CD90, CD73 and CD44, to a similar level than their counterparts grown on 2D tissue culture plastic (TCP) suggesting that the cells retained their stem cell phenotype. We further showed evidence of multilineage differentiation of hMSCs towards osteogenic, adipogenic and chondrogenic lineages within PEGDA SPHs and confirmed that long-term culture did not induce auto-differentiation. Thus, we conclude that architecture and physicochemical characteristics of a TE scaffold can impact stem cell behavior and lineage progression. These scaffolds show great promise in regenerative medicine and might also be used as model 3-D systems for studying cell behavior in response to various stimuli.

Based upon these conclusions and our past observations, future directions of this work may focus on the identification of serum proteins that mediate initial hMSC attachment. The identification of proteins that control this process would help in the design of new materials for improved cell attachment or serum-free MSC culture. In our studies the ECM component laminin was being elevated in the SPH cultured hMSCs. Laminin is bound by several integrin receptors, all of which are expressed in hMSCs, suggesting that laminin might play a role in adhesion of hMSCs to PEGDA SPHs and their end fate. Mass spectrometric analysis further revealed a different panel of adsorbed proteins for nonporous PEGDA gels and SPHs suggesting that adsorption was a directed process (study conducted by Samuel Erb).

Typical TE scaffolds are degradable *in vivo*. The TE construct degrades and gets remodeled by new tissue as cells migrate into the scaffold and synthesize new ECM. Crosslinked PEGDA hydrogels are not degradable under physiologic conditions. Thus, future studies should be directed towards creating degradable SPHs and to compare tissue response and vascularization with non-degradable systems. Degradable PEG-based SPHs can be synthesized by the incorporation of metalloproteinase (MMP)-sensitive peptides into the polymer backbone (Lutolf and Hubbell, 2003).

Our observations within the SPHs indicated a discrepancy between mineralization and gene expression data. The increased calcium levels in the osteogenic induction group were not followed by an upregulation of osteogenic gene markers. Thus, we evaluated putative adipogenic and osteogenic marker genes for their specificity towards the respective lineage (chapter 3). We identified fatty acid binding protein 4 as specific adipogenic gene marker. But many of the markers used for determining the end fate of differentiated cells were shared between adipogenic and osteogenic differentiated hMSCs. All osteogenic marker genes tested were also expressed in adipocyte-derived MSCs. Alkaline phosphatase and osteopontin were even significantly upregulated during adipogenic differentiation. Thus, one should be cautious when using these markers to indicate bone formation. Our data indicate the need for a better understanding of the molecular mechanisms involved in controlling the differentiation of hMSCs as well as the plasticity between mesodermal lineages. This knowledge is essential to manipulate adult stem cells for engineering functional tissues in regenerative medicine. We further showed that reference genes involved in the carbohydrate mechanism (GAPDH) and ribosomal proteins (RPL13α) can produce altered gene expression patterns during osteogenic differentiation.

Future research should involve microarray experiments to identify markers that are more exclusive to their respective tissue-specific lineage and eventually also predict the *in vivo* response. Further, dual expression of adipogenic and osteogenic markers by immunofluorescence staining on a single cell level can give answers if the expression of certain markers is indeed shared between both lineages or due to the heterogeneous nature of stem cell cultures. The Wnt signaling pathway follows an inverse balance beween osteogenic and adipogenic differentiation and generally provides proosteogenic/antiadipogenic stimuli. It has been demonstrated that ß catenin encourages the progression of MSCs from osteoblastic precursor cells into mature osteoblasts while suppressing adipogenic and chondrogenic differentiation (Case et al., 2010). Future research on stem cell differentiation markers might also include investigations of the Wnt signaling pathway.

Another major obstacle to the clinical application has been the establishment of vascular structures within cell-based TE constructs for the supply of blood and oxygen. Especially constructs greater than 1 cm in thickness cannot rely solely on the ingrowth of host vessels to stay viable *in vivo* (Davis et al., 2007). An appealing strategy to overcome this limitation is to create microvascular structures within the engineered scaffold itself. Thus, cells with vessel forming capabilities that can be expanded in large numbers are required.

In previous chapters, we confirmed the adipogenic, osteogenic, and chondrogenic differentiation potential of hMSCs on TCP and within PEGDA SPHs, a prerequisite for functional tissue formation. In chapter 4, we further investigated hMSC differentiation towards the endothelial lineage and evaluated the prevascularization potential of endothelial-like hMSCs (EMSCs) *in vitro* within SPHs and the angiogenic response *in vivo*.

With the differentiation protocol established during this dissertation research it is still

unclear whether "true" endothelial cells can be obtained since we did not observe a significant upregulation of the endothelial marker CD34 and EMSCs also did not acquire the cobblestone morphology typical for endothelial cells. However, we showed that hMSCs gain some phenotypic and functional features of endothelial cells as indicated by the expression of CD309 and the formation of tube-like structures on matrigel and within collagen-filled SPHs. It is suggested that EMSCs contribute to the formation of a prevascular network within SPHs. Due to the accessibility and their proliferative capacity, MSC-derived endothelial cells may qualify for numerous TE applications in the future.

After implantation onto the chick chorioallantoic membrane (CAM) for 7 days, we observed lumen-like structures to a similar extent in cell-free and in prevascularized constructs. Confirming previous studies (Keskar et al., 2009b), SPHs alone without the presence of cells promoted the ingrowth of avian CD34 positive endothelial cells suggesting that the porous architecture of the scaffold is an important factor in the revascularization process of these scaffolds *in vivo*. However, collagen-containing vascularized tissue was only present in constructs that were preseeded with cells and most prevalent in EMSC- and HUVEC-containing scaffolds indicating the potential of the prevascularization approach. The ideal biomaterial replaces normal tissue without inducing a foreign body response that eventually leads to rejection or necrosis. None of our tested constructs induced a mature foreign body reaction. Only mild inflammatory responses were detected such as the increase in thickness of the stroma and squamous metaplasia of the ectoderm at the scaffold interface indicating the suitability of PEGDA SPHs for TE applications.

Further studies should be conducted to evaluate vessel ingrowth into preseeded scaffolds at earlier timepoints. In general, in TE settings a rapid vascularization of the scaffold after

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implantation is desired to promote cell survival. The prevascularization approach has been shown to reduce the critical time of early vascularization and to ensure a rapid life-sustaining anastomosis with the host endothelium. For instance, in a prevascularized fibrin gel, vessels from the host could be detected 5 days after implantation whereas the same process took 14 days in the non-prevascularized gel (Chen et al., 2009b). When a high density of fibroblasts was coseeded with HUVECs in the same TE construct, the ingrowth of host vasculature could be accelerated by 2 or 3 days (Chen et al., 2010).

Engrafted endothelial cells (ECs) and the ingrowing host vasculature connect in a process called wrapping-and-tapping (WAT) anastomosis (Cheng et al., 2011). ECs within the TE construct wrap around the ingrowing host vessels, and cause degradation of the host endothelium to redirect blood flow to the engineered vascular network. High expression levels of MMP-14 and MMP-9 accompany this process. Thus, proper manipulation of local MMP levels might lead to faster perfusion of TE constructs. Various approaches could be exploited: MMP delivery systems could be incorporated into the scaffold or MMP-peptides could be structurally incorporated into the biomaterial.

We showed that MSCs are able to undergo osteogenic differentiation within our SPHs. Bone is highly vascular and interactions between ECs and osteogenic progenitors are critical for successful bone development (Deckers et al., 2002; Kaigler et al., 2003). ECs enhance the proliferation and differentiation of osteoprogenitor cells through the secretion of osteogenic growth factors such as bone morphogenetic proteins (BMPs) (Shin et al., 2004). On the other hand, MSCs are known to secrete VEGF and thus stimulate EC proliferation (Deckers et al., 2000). Thus, future research can involve the investigation of most favorable cell

combinations of osteogenic and endothelial progenitors, cell ratios and optimal culture conditions that allow the different cell types to perform simultaneously.

In chapter 4 we encapsulated one cell type at the time in collagen gel and seeded the cell suspension onto the SPH. We could further exploit the features of the porous hydrogel scaffolds by encapsulating cells within the hydrogel matrix of the SPH and seeding another cell type within the porous network (Scheme 5.1). This would allow spatial separation of different cell types and cell development could occur more independent from each other. For instance, encapsulated MSCs could be induced towards the osteogenic lineage. After osteogenic differentiation, MSCs can be seeded into the porous network and differentiated towards the endothelial lineage. Paracrine signaling between the two cell types may contribute to enhanced tissue formation.

To recapitulate, we identified a suitable scaffold for stem cell delivery and demonstrated that the macroporous architecture of the scaffolds improves cellular functions and responses. We confirmed the adipogenic, osteogenic, chondrogenic and endothelial differentiation potential of hMSCs within PEGDA SPHs and showed the benefit of hMSCs in vascularization approaches. We further demonstrated the need for more definite differentiation markers to better confirm the specificity of the differentiation program of MSCs.

Scheme
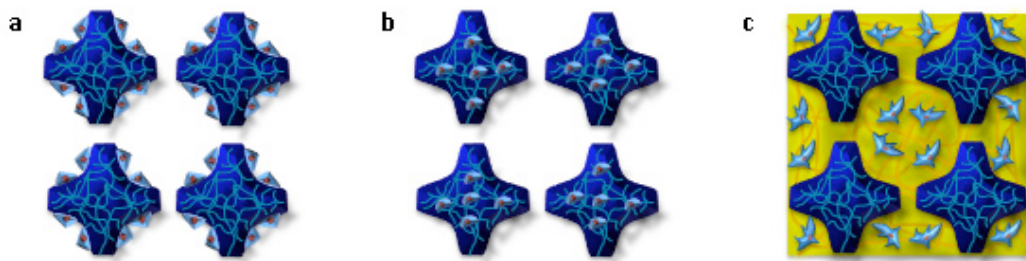
Scheme 5.1 Various ways of cell delivery within SPHs. (a) Cells can be seeded within the porous network of the SPH, (b) cells can be encapsulated within the hydrogel network, and (c) cells can be embedded in collagen and seeded within the porous network of the SPH.

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6 Appendix

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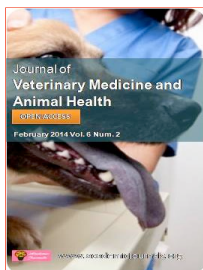
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Published February 2014 - **Vol. 6 Num. 2**

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VITA

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- Studied the survival, trilineage differentiation potential and extracellular matrix deposition of adult mesenchymal stem cells (MSCs) within poly(ethylene glycol) diacrylate (PEGDA) superporous hydrogel scaffolds to assess the potential of these constructs in regenerative medicine.
- Established real-time PCR assays to evaluate adipogenic and osteogenic marker genes for their specificity towards the respective lineage.
- Assessed the *in vitro* endothelial differentiation potential of MSCs using flow cytometry and the tube formation assay.
- Introduced the chick chorioallantoic membrane assay to the laboratory and evaluated the angiogenic potential of endothelial-like MSCs in superporous hydrogel scaffolds.

Research Project with Roquette America Inc. (Collaborator: Dr. Carmen Popescu) — 2012
- Evaluated the stability of benzocaine in commercially available oral disintegrating tablet (ODT) platforms according to ICH guidelines. LCMS was used to monitor benzocaine degradation over 1 year.

Visiting Research Specialist, Department of Biopharmaceutical Sciences, UIC, Chicago, IL — 2007
- Carried out drug release studies from polycaprolactone (PCL) nanofibers.
- Conducted cytotoxicity tests of camptothecin-loaded PCL nanofibers on U-87 MG cells.

Research Intern, Department of Pharmaceutical Chemistry, Philipps University, Marburg,   2005
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- Cloned the Escherichia coli rnpB gene and the Bacillus subtilis rnpB gene into different plasmids to study the interchangeability of type A and type B Rnase P RNA.

Teaching Experience

Teaching Assistant, Department of Biopharmaceutical Sciences, UIC, Chicago, IL   2008-2010
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Students supervised

Melody Lee, Undergraduate Student, Biology (Honors College), UIC, Chicago, IL	2009
Adrian Stecula, REU Student, Chemical Engineering, University of Michigan, MI	2010
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Member, Rho Chi Honor Society	2010
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Member, Controlled Release Society Student Chapter (CRS-IL), UIC	2008
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Awards

1st place at poster competition in the predoctoral (PharmD, PhD, or MS) category at the College of Pharmacy Research Day	2010
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UIC Graduate Student Presenter Award	2011
Lundbeck Award for Excellence in Research	2012
W.E. van Doren Scholar	2013
Roquette America, Inc. Student Scholarship	2013

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6. Erb SJ, **Köllmer M**, Lee BS, Gemeinhart RA. Serum Proteins mediate Cell Attachment within Poly(ethylene glycol) diacrylate (PEGDA) Superporous Hydrogels, College of Pharmacy Research Day, Chicago, IL (February 2011).
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16. **Köllmer M**, Popescu C, Manda P, Zhou L, Gemeinhart RA. Benzocaine Degradation in Oral Disintegrating Tablet Platforms. AAPS Annual Meeting, San Antonio, TX (November 2013)