MATRIX SUPPLEMENTED STEM CELL MICROENCAPSULATION FOR REGENERATIVE MEDICINE

By

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Previous studies reported that matrix protein supplementation (fibronectin/fibrinogen, FN/FG) of agarose gel microcapsules enhances survival and pulmonary retention of syngeneic rat multipotent stromal cells (MSCs). I hypothesized that additional supplementation of microcapsules with osteopontin (OPN) and transglutaminase 2 (TG2) would enhance cell survival, while stabilizing the provisional matrix. Using monomeric OPN or OPN polymerized with TG2, I examined human MSC adhesion, morphology, focal contact formation and apoptosis. Polymeric OPN induced greater adhesion than monomeric OPN (84.5±10.7 vs. 44.3±10.0cells/field), and also significantly enhanced focal contact formation (351.5±21.2 vs. 45.6±17.6 focal contact sites/cell) and cell spreading ($2.7x10^3±0.20x10^3 \mu m^2$ vs. $1.2x10^3±0.26x10^2 \mu m^2$) while preserving MSC pluripotency. Microcapsules supplemented with FN/FG, polymeric OPN and TG2 demonstrated significantly less apoptotic cells than FN/FG microcapsules (14.0±2.34% vs. 28.2±3.22%). Reduced apoptosis was attributed to matrix stabilization by TG2 and the synergistic activity of matrix proteins. It is anticipated that this enhanced survival will maximize the therapeutic potential of MSCs.

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Abbreviations

αMEM	Alpha minimum essential media
BSA	Bovine serum albumin
CCM	Complete culture media
CCN	Cyr-61, CTGF [connective tissue growth factor], Nov
CMTMR	5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl rhodamine)
DMEM	Dulbecco's modified eagle's medium
DMPS	Dimethylpolysiloxane
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FABP-4	Fatty acid protein-4
F-actin	Filamentous actin
FBS	Fetal bovine serum
FN	Fibronectin
FG	Fibrinogen
FGF	Fibroblast growth factor
HBSS	Hank's balanced salt solution
hMSC	Human multipotent stromal cell
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
IGF-1	Insulin-like growth factor-1
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MMP	Mitochondrial membrane permeabilisation
MSC	Multipotent stromal cell
NF-κB	Nuclear factor κB
NO	Nitric oxide
OPN	Osteopontin
OPN-poly	Polymeric osteopontin
PBS	Phosphate buffered saline
PI-3K	Phosphoinositide-3-OH kinase
PKB	Protein kinase B
Poly-Hema	Poly (2-hydroxyethyl methacrylate)
PVDF	Polyvinylidene fluoride
RGD	Arginine-glycine-aspartic acid recognition motif
SPARC	Secreted protein, acidic and rich in cysteine
STRO-1	Stromal cell derived factor-1

TG	Transglutaminase
TG2	Transglutaminase 2
TSP1	Thrombospondin 1
TSP2	Thrombospondin 2
TNFR	Tumor necrosis factor receptor
VEGF	Vascular endothelial growth factor

1.0 Introduction

Tissue specific stem cell therapy has exciting potential to regenerate damaged tissue and restore organ function. The capacity of stem cells to home to sites of injury, differentiate into distinctive cell types, and promote the formation of regenerative microenvironments forms the foundation for Regenerative Medicine and has fuelled interest in clinical tissue regeneration. However, simple cell injection results in very low overall cell survival and poor organ-specific cell retention that has greatly limited the success of cell-based therapies to restore functional tissue[1-3]. This thesis presents an innovative cell-delivery strategy designed to improve cell engraftment and viability that are needed to realize the maximum potential of cell based therapies.

1.1 Regenerative Cell Therapies

There has been considerable interest in the use of adult stem cells from bone marrow stroma[4-7], referred to as multipotent stromal cells or marrow stromal cells (MSCs), for cell therapy applications. Human stem cells can be readily acquired from a small aspirate of bone marrow and demonstrate high expansion potential while preserving their ability to differentiate into multiple cell lineages including adipocytes[8], osteoblasts[9], chondrocytes[10]. As well, these cells can exhibit phenotypic qualities of endothelial[4], neural[11], muscle[12], skeletal myoblasts[13] and cardiac myocyte cells[14-18]. Human MSCs may represent an ideal candidate for cell-based therapies as they are a robust, multipotent cell type that exhibit genetic stability[19]. Moreover, there is also considerable evidence that allogenic MSCs can be used for cell therapy, since they appear they may be immune priveledged and thus do not pose risks of immunorejection[20,21]. MSCs delivered by intravascular injection have the capacity to home to sites of injury and promote tissue repair and can also be employed as vectors for delivery of therapeutic genes[22]. Consequently, considerable interest has been directed toward the biology and therapeutic potential of these cells as possible agents for regenerative cell therapies.

1.1.1 Multipotent Stromal Cells

Initially described by Friedenstein et al., MSCs are fibroblast-like cells residing in adult tissues that can be isolated, expanded in culture and characterized *in vitro* and *in vivo*[23]. These spindle-shaped, plastic-adherent cells are primarily isolated from bone marrow, but are also present in adipose tissue[24], circulating blood[25,26], cord blood[27,28], placenta[29], amniotic fluid[30], heart[31], skeletal muscle[32], synovial tissue[33], pancreas[34] as well as numerous other tissue sources. Bone marrow-derived MSCs are the most extensively characterized form of this stem cell population and majority of the understanding of their multipotency has been based on differentiation studies of marrow-derived MSCs[21].

Human MSCs are traditionally isolated from the low-density mononuclear cell population of bone marrow based on their selective adhesion to plastic, unlike hematopoietic cells[25,35,36]. The adherent stem cell population is cultured at low passage and has been assessed for the expression of cell surface markers by flow cytometry. Although there is a lack of specific markers characterizing multipotent MSCs, the cells are negative for hematopoietic antigens: CD34, CD45, CD14 and positive for numerous markers including STRO-1, CD29, CD73, CD90, CD105, CD166 and CD44[37-39]. The resulting population isolated is heterogeneous in relation to differentiation potential among single cell clones of MSCs, and *in vitro* differentiation studies have shown that approximately 30% of the clonal MSC are multipotent and thus true "stem cells"[38,40]. However, due to the lack of specific markers distinguishing multipotent MSCs, functional assays are often employed to demonstrate the capacity of these cells to readily differentiate to mesoderm-type cells (adipocytes, osteocytes and chondrocytes) under appropriate *in vitro* conditions[10,38,41].

1.1.2 Multipotent Stromal Cell Therapy

MSC therapy has shown promise both in experimental and clinical studies. Over 300 patients have received systemically infused MSCs for various pathological conditions[42]. Safety of MSC transplantation was initially demonstrated in a phase I trial in 1995, where bone marrow samples from patients with hematologic malignancies were collected, MSCs were then expanded in *ex vivo* culture and administered through intravenous infusion[43].

Transplanted cells did not exhibit any toxicity and it was hypothesized that MSCs would home to the marrow and rebuild the surrounding microenvironment. More recently, MSCs or bone marrow mononuclear cells have been administered to patients with vascular ischemia secondary to peripheral arterial disease[44], coronary artery disease[45-47] or non-healed chronic skin wounds[48]. Cell administration was well tolerated and in many cases restored microvascular function and promoted remodeling in sites of injury of the respective studies. Numerous preclinical studies in various animal models have demonstrated a healing response induced by engraftment of MSCs in damaged myocardium,[21,49] however, the underlying therapeutic mechanism of the beneficial effects of MSCs remains unclear.

Some groups have suggested that remedial effects in the myocardium induced by MSC infusion may be attributed to a multitude of mechanisms; including matrix reorganization, transdifferentiation, immune modulation as well as paracrine activity[50-53]. MSC implantation in the damaged myocardium potentially improves pathologic remodeling due to changes in cellularity and extracellular matrix remodeling[20]. This reorganization in the damaged tissue is believed to contribute to the remedial effects noted following MSC infusion, those being a more compliant, less stiff ventricle with improved diastolic-filling properties[20]. The multipotency of MSCs is also thought to contribute to their therapeutic potential. Although, MSC capacity to transdifferentiate into cardiomyocytes is a highly controversial topic, some groups have suggested that engrafted MSCs display cardiomyocyte phenotype with the expression of myogenic markers[54]. Other groups have argued that the extracted MSC population capable of differentiating into a cardiac lineage may be contaminated with hematopoeitic stem cells[55]. Immunomodulatory capacity of MSCs has also drawn attention as a potential therapeutic mechanism employed by these cells. MSCs have the ability to induce apoptosis of activated T cells and may also regulate dendritic cell differentiation, maturation and function[21]. Recent studies have also suggested that the paracrine effects of administered cells may also play an important role[56,57]. Administration of MSCs in ischemic rat heart resulted in increased production of angiogenic factors, such as basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and stem cell homing factor (stromal cell-derived factor -1α), coupled with decreases in proapoptotic protein Bax[57]. This evidence of paracrine activity was also accompanied by increases in capillary density and improvement

in cardiac performance[57]. All together these potential therapeutic mechanisms may function to support a regenerative microenvironment and thereby repair damaged tissue.

To date systemic delivery of MSCs appears to be a safe, non-invasive and a clinically relevant approach for cell therapy. Studies examining the fate of systemically infused MSCs performed in a murine model demonstrated that gene-marked cells administered by tail vein injection were present in the marrow, spleen, bone, lung and cartilage[58]. Employment of numerous methods for quantification of MSC engraftment in the heart post-cell delivery has revealed very modest cell engraftment in all studies. Some studies have shown that less than 3% of MSCs delivered by direct injection are present after two weeks, and administration of increasing numbers of cells only results in a modest increase in long-term persistence[20]. Other animal studies have shown that direct injection of cells into the myocardium results in an immediate loss of approximately 40% of the injected cells. Initial loss of cells can be attributed to leakage from the injection site or washout via the vasculature or lymphatic system[3,59]. A wave of apoptotic cell death persisting for up to 4 days follows the initial cell loss[60]. The net result is a minimal retention of viable cells and a limited regeneration of functional tissue. In order to address these challenges, it is important to understand the underlying causes of transplanted cell death.

1.2 Cell Viability & the Extracellular Matrix

Efficacy of cell transplantation is limited in part by a form of anchorage-dependent apoptosis known as anoikis[61,62]. This occurs when cells are put into suspension due to the detachment of anchorage-dependent cells from the surrounding extracellular matrix (ECM), resulting in loss of cell-matrix signaling and ultimately cell death. Laflamme et al. (2007) demonstrated in a recent study that human embryonic stem cell derived cardiomyocytes require a prosurvival supplemented matrix to promote survival, engraftment and functional improvement of the infracted myocardium[62]. Although the authors noted that the increase in cell survival was multi-factorial, inhibition of anoikis was a prominent factor[62]. Low levels of cell survival is hindering in the development of effective cell therapies for several pathological conditions, including diabetes[63,64], Parkinson's disease[65,66], muscular dystrophy[67,68], and acute myocardial infarction[62]. A better understanding of apoptosis and

more specifically anoikis in the context of cell transplantation may lead to better ways to overcome this critical limitation.

1.2.1 Apoptosis

Programmed cell death, termed apoptosis, is derived from the Greek word meaning 'dropping off' or 'falling off' as leaves from a tree, is a mechanism of controlled cell desctruction that is essential for the maintenance of tissue homeostasis (for review see [69]). In response to pro-apoptotic signals (or lack of appropriate survival cues), an intracellular mechanism is activated to eliminate unneeded, damaged or infected cells. Apoptotic cells display a sequence of distinct morphological changes including cytoplasmic shrinkage, membrane blebbing and eventual fragmentation, condensation of chromosome and DNA fragmentation[70-72]. Small membrane bound apoptotic bodies are released following membrane fragmentation and these are generally engulfed by other cells. These well characterized morphological changes are distinct from those of necrotic cells which typically swell and burst in response to tissue damage. The sequence of morphological changes displayed by apoptotic cells proceeds concomitantly with a series of signaling events highly conserved among numerous species. These include activation of a family of cysteine proteases known as caspases which function to cleave other caspases that subsequently cleave short amino acid sequences of target proteins such as those of the nuclear lamina and cytoskeleton, thereby leading to the destruction of the cell[70-72]. Although caspases are critical players in many apoptotic events, caspase-independent programmed cell death is also prevalent. In the absence of caspase activity, perturbations in cell metabolism, growth factor availability and genotoxic agents are sufficient to stimulate apoptosis[72]. Sensory proteins such as BH3-only proteins function to transmit appropriate signals to multidomain proteins such as Bax and Bak which thereby integrate these signals to commit cells to an apoptotic fate[73]. Outer mitochondrial membrane permeablizations follows, releasing caspase activating factors, including cytochrome c, and thereby resulting in cell termination. This sequence of events is triggered by factors intervening in BH3-only protein expression or activity. These factors include DNA damage, growth factor receptor signaling, extracellular death ligands such as FasL and Tumor Necrosis Factor Receptor (TNFR) as well as cell adhesion machinery, integrins[73-78].

1.2.2 Anoikis

Anoikis, derived from the Greek term describing '*homelessness*', is induced by the loss or lack of appropriate cell-matrix adhesion. Cells interact with their surrounding microenvironment to form anchorage sites through bidirectional interactions between various extracellular components and the intracellular cytoskeletal network. Initially discovered in epithelial and endothelial cells, experimental dissociation of these cells from their surrounding extracellular matrix demonstrated morphological changes, internucleosomal DNA cleavage and nuclear lamin cleavage that were characteristic of apoptosis rather than necrosis[79,80]. Cellular commitment to this experimentally induced apoptosis could be reversed by re-establishing cell-matrix interactions within a short time of dissociation.

The process of anoikis encompasses a broad array of cellular responses due to many different forms of cell-matrix interactions, and is therefore, the end-stage product of numerous signaling pathways rather than being defined by a single molecular mechanism resulting in apoptosis[81]. Universal regulators of anoikis, however, are cell surface receptors of the integrin family. Cells plated in suspension culture conditions with immobilized integrin-activating antibodies do not succumb to anoikis, while immobilized antibodies targeting other cell surface receptors induce anchorage-dependent cell death[79,80]. Integrin-mediated cell survival signal transduction pathways play a key role in maintaining cell viability. These pathways include kinase signal transduction pathways of mitogen-activated protein kinase (MAPK)/ras-extracellular signal regulated kinase (ERK) pathway and the phosphoinositide-3-OH kinase (PI-3K) pathway and its downstream target protein kinase B (PKB or AKT)[82,83]. Overexpression of Bcl-2 has also been shown to inhibit anoikis, suggesting an importance of mitochondrial membrane permeabilisation (MMP) occurrence[79,80].

Expression levels of Bcl-2, bax and related proteins have been correlated with the sensitivity of cells to anoikis, a cell-type specific characteristic[79,80]. Fibroblasts can resist the onset of anoikis in the absence of ECM, provided that serum growth factors are present, while epithelial and endothelial cells demonstrate greater sensitivity to anoikis[84]. MSCs,

similar to epithelial and endothelial cells, are anchorage-dependent cells interacting with their surrounding microenvironment such that it plays a paramount role in survival and function, and thus MSCs are also sensitive to anoikis[85].

1.2.3 Cell-Matrix Interactions

Modifying the microenvironment surrounding adherent cell populations to promote appropriate ECM interactions involving numerous integrin mediated intracellular signaling molecules can prevent the onset of anoikis[86,87]. The ECM is a complex network of proteins and polysaccharides surrounding cells and comprising the physical scaffold on which cells reside. The intricate microenvironment is secreted and assembled by the surrounding cells and functions as the framework for cell adhesion and migration. Associations between cells and ECM provide cues concerning a cell's milieu within a tissue or organ, and thereby mediate crucial signaling pathways affecting cell proliferation, migration, differentiation and survival[88].

Interactions between cells and the surrounding matrix are primarily mediated by integrins, and thus these transmembrane receptors play an important role both as anchorage machinery and as regulators of anoikis. Integrin heterodimers are composed of non-covalently associated α and β subunits. Each integrin subunit is comprised of an extracellular head and a cytoplasmic tail, in whole functioning as transmembrane mechanical links between extracellular contacts and the cytoskeleton. With the exception of α6β4, all integrins associate with the actin-based microfilament system and thereby regulate and modulate this cytoskeletal network[89]. The cytoplasmic domain of the β4 subunit is approximately 20 fold longer than other β subunits and in turn connects to intermediate filaments, unlike all other β subunits[89]. Extracellular integrin heterodimers bind to specific amino-acid sequences such as the arginine-glycine-aspartic acid (RGD) recognition motif present in many matrix proteins, including fibronectin, fibrinogen, osteopontin and vitronectin[88,90]**.** Following extracellular substrate recognition and binding, integrins travel laterally in the plain of the cell membrane to construct clusters referred to as focal adhesion (also known as focal contact) sites. These concentrated signaling centres reinforce substrate adhesion and localize actin filaments and signaling constituents[91]. Focal adhesion sites and the cytoskeletal network establish the

structural framework around which cell morphology and polarity take shape as well as provide the dynamic properties required for cell migration and division[91].

Anchorage of the cell membrane by integrins stimulates signaling pathways governing cell survival, migration, cell cycle progression as well as expression of differentiated phenotypes[92]. Pathological states, including blood clotting, wound healing defects and malignant tumour formation are often associated with abnormalities in integrin binding interactions, thereby reaffirming the central role of integrins in development, organization, maintenance and repair of numerous tissues[93]. Fibroblasts lacking functional integrin-linked kinase (ILK), a binding partner of the cytoplasmic domain of integrins and pivotal effector in the transduction of signals from the ECM, display dramatically impaired cell spreading, F-actin aggregation, focal adhesion formation and proliferation[94]. Gronthos et al. (2001) demonstrated that human MSCs exhibit higher colony-forming efficiency in the presence of collagen type IV, fibronectin, vitronectin and laminin in comparison with collagen type I and III[95]. Differential growth patterns were attributed to a combination of factors including integrin expression, which was reaffirmed when the ability of MSCs to differentiate into osteoblasts was significantly stunted in the presence of β1 integrin blocking antibody, thereby pointing to an important role for β1 integrins in MSC differentiation. Thus, engagement of integrins by their surrounding microenvironment leads to a cascade of signaling events governing cell function. However, both the physical state and the molecular composition of the surrounding matrix are also crucial in determining the signal transduction pathways activated and the magnitude of their activation[96-98].

By employing integrins as "sensors", cells respond to the surrounding matrix in specifc ways depending on its composition, ligand immobilization along with physical properties such as rigidity. Tissue stiffness in the human body varies considerably, with approximately three orders of magnitude difference between the softest and stiffest tissues, i.e. brain and bone respectively[98]. Cells of these tissues are able to respond to the static mechanical environment appropriately with focal adhesions and integrins as the primary method of communication between the ECM and cytoskeleton. The cytoskeletal network is organized based on the traction forces exerted by cells in response to the matrix. The balance of forces

manifested in the cytoskeleton are dependent on the rigidity of the surrounding matrix, activation of actomyosin motors and tension within the cytoskeleton[99]. This cytoskeletal arrangement dictates cell morphology as well as signaling pathways governing migration, proliferation and differentiation. Rigid ECM substrates are capable of supporting stable cellular force balances, where disruption of multiple stress fibers displays negligible changes in cell shape and cell-matrix interactions[99]. On the other hand, disruption of a single stress fiber of cells cultured on flexible ECM substrates displays cytoskeletal rearrangement, retraction of cell-matrix associations as well as dramatic changes in cell morphology[99]. Katz et al. (2000) proposed that the molecular organization of adhesion domains are dictated by two important factors; first integrin-ligand binding specificity contributes to the organization of respective cytoplasmic anchor proteins, and second, ECM rigidity modulates tension localized at adhesion sites and promotes tyrosine phosphorylation and thereby recruiting various plaque molecules at adhesion sites[97]. These components of molecular organization result in structurally and functionally distinct forms of matrix adhesions, and in turn, contribute to the array of signaling pathways that are stimulated leading to different cellular responses.

1.2.4 Matrix Crosslinking

Matrix rigidity is largely dependent on chemical crosslinking of constituent proteins. Depending on the crosslinking agent, matrices can be further stabilized by inducing the formation of covalent bonds between substrate proteins. These post-translational modifications alter the mechanical properties of the ECM, while modifying the conformation of the constituent proteins such that they induce cellular responses distinct from those of non-crosslinked proteins[100,101]. Crosslinking agents such as transglutaminases (TG) represent a family of 9 known members, including the plasma TG, factor XIII, TG1 (keratinocyte TG), TG2 (tissue TG), TGs 3-7 and erythrocyte band 4.2 protein[102]. All TGs, with the exception of erythrocyte band 4.2 protein, catalyze the formation of high molecular weight complexes of their substrate proteins by forming isopeptide crosslinks from glutamine and lysine residues and releasing ammonia[102]. Resulting covalent bonds between matrix proteins are stable and resistant to proteolysis, and the presence of these stabilized matrices enhances the durability and integrity of tissue.

TGs have also been implicated in cell adhesion, spreading and signaling, in addition to matrix stabilization, wound healing, bone formation, hemostasis, along with other roles in vascular biology[102-107]. Although much remains to be learned about the physiological roles and expression of many of the TGs, it is known that TG1, TG2 and factor XIII are expressed in the vascular system[107]. TG1 expression has been noted in cultured endothelial cells, as well as myocardial endothelial cells in mice, however, lung endothelial cells in mice do not display TG1 expression[108,109]. It has been suggested that TG1 may serve to support intercellular junctions and barrier functions of myocardial endothelial cells through stabilization of the actin filament network[108,109]. Factor XIII and TG2 have been more widely studied than TG1. Factor XIII is present in the vascular system both as a heterotetramer and as a homodimer. As a heterotetramer, factor XIII circulates in plasma, while it exists as a homodimer in platelets and cells derived from monocytes/macrophages present in the blood vessel wall[107]. Enzyme subunits derived from cells in either the bone marrow or liver[110] are assembled into an intact enzyme, which requires thrombin cleavage and Ca^{2+} for its activation[102]. As a major contributor to hemostasis and wound healing, factor XIII has recently been reported to play an important role in the healing of infarcted myocardium[111]. Factor XIII-deficient mice developed lethal left ventricular rupture within a few days post myocardial infarction., which could be rescued by the administration of intravenous factor XIII[111]. TG2 on the other hand is ubiquitously expressed in the vasculature, primarily due to its presence in endothelium and smooth muscle cells[112]. However, it is also expressed in fibroblasts and monocytes/macrophages[107]. Expression of TG2 has been noted in all layers of the vessel walls of small arteries from rats, mice and pigs, with pronounced presence in the endothelium[113,114]. Although a small percentage of cells secrete TG2 into their surrounding microenvironment, TG2 is predominately present in the cytosol[102]. This Ca^{2+} dependent enzyme displays colocalization with cytoplasmic stress fibers[115], whose formation may be partly attributed to TG2 activity[116,117]. In addition, as an intracellular enzyme, it has been proposed that TG2 may serve to stabilize intracellular proteins following the onset of apoptosis[118]. Prior to the complete dismantelling of the apoptotic cell, deviations from cellular homeostasis leading to increases in intracellular Ca^{2+} concentration may activate chemical crosslinking of intracellular proteins[107,118]. In the extracellular space, TG2 interacts

with β1 and β3 integrins in focal adhesion sites by functioning as a bridge between respective integrins and ligands including fibronectin[119]. These stabilized associations reinforce and enhance cellular interactions with surrounding matrix proteins[119]. In addition to serving as an adhesion co-receptor, TG2 can crosslink osteopontin, an adhesive glycoprotein part of a specialized group referred to as matricellular proteins, resulting in enhancement in its activity, mediated by conformational changes, exposing cryptic integrin binding sites and thereby enhancing cell-matrix interactions[101,120]. Together with the ability of TG2 to stabilize cell-matrix adhesion sites, these effects are of considerable interest for enhancement of cell anchorage in cell-based therapies, which has shown to be a key limitation.

Overall, TGs have the capacity to stabilize matrices and generate rigid structures capable of heightening cell-matrix interactions. Catalytic activity of TG2, in particular, enhances the biological activity of proteins modulating cell-matrix interactions (osteopontin, a matricellular protein) through protein crosslinking. Thus, anchorage dependent cells interacting with rigid matrices comprised of TG2-crosslinked proteins may in turn be protected from anoikis through stabilized adhesion sites, subsequently leading to heightened activation of cell survival signaling pathways. Therefore, matrix crosslinking agents such as TG2 may potentially be useful as tools in conjunction with substrate proteins to enhance the efficacy of cell based therapies.

1.2.5 Matricellular Proteins

The term 'matricellular' encompasses a group of ECM proteins that modulate cell function without serving structural roles like traditional matrix proteins[121]. This group of proteins includes thrombospondin-1 (TSP1), SPARC (secreted protein, acidic and rich in cysteine; also referred to as osteonectin), tenascin-C, tenascin-X, periostin, TSP2, osteopontin and a few other proteins such as the CCN (cyr-61, CTGF [connective tissue growth factor], Nov) family of proteins, remain under consideration as members of this group[122-124]. A commonality between all matricellular proteins is their high expression during embryogenesis, followed by a steep decline after birth[121]. In the adult, matricellular protein expression is generally limited to regions of tumor growth and tissue injury[121]. These expression patterns have been suggested to correlate with roles for these proteins in

enhancing the migratory and proliferative capacity of malignant and wound healing cells, along with functioning in the remodeling of the ECM, potentially through alterations in the adhesive activity of cells and modifications in proteinase activity, respectively[121,125].

Matricellular proteins have the capacity to bind cell surface receptors, including integrins, and regulate the expression and activity of growth factors, cytokines, and proteases, in turn allowing these proteins to modulate cellular function and matrix production[121,125]. Periostin, a recent addition to the matricellular protein family, demonstrates striking ability to modulate cellular function by engaging α_v, β_1, β_3 and β_5 integrins of cardiomyocytes[126]. Administration of exogenous periostin into infarcted myocardium resulted in dramatic functional and remodeling improvements of the myocardium along with reduced fibrosis and infarct size and increased angiogenesis[126]. Therapeutic potential of extracellular periostin was attributed to its ability to induce proliferation of resident cardiomyocytes[126]. However, other matricellular proteins, including TSP2 and osteopontin, also have interesting properties thay may be useful in cell therapy applications.

The physiological role and thereby the therapeutic potential of matricellular proteins has been investigated using knockout mice in which these proteins were disrupted by gene targeting. Interestingly, all matricellular protein knockout mice survived embryogenesis, yet exhibited dramatic abnormalities in wound healing and matrix remodeling post injury in adult mice[127]. These results further emphasize the pivotal role of these proteins in normal tissue healing and remodeling[127]. OPN null or TSP2 deficient mice showed distinct phenotypes post myocardial infarction. More than 90% of TSP2 null mice succumb to cardiac rupture 48 hours after myocardial infarction[128]. Thus, TSP2 may play an important role in left ventricular remodeling post myocardial infarction, and may also serve to maintain matrix integrity in the heart. In contrast, OPN gene deficient mice exhibited exaggerated left ventricle dilatation, and decreased collagen deposition in both the infracted region and surrounding area of the heart following myocardial infarction[129]. Up-regulation of OPN following myocardial infarction prevented severe tissue damage by protecting against left ventricular dilation, and thereby implicating an important role of OPN in managing cardiac injury. In response to cardiac injury or stress, pronounced expression of matricellular proteins

is noted in the heart and may mediate cardiac remodeling either through its functions in cell-matrix interactions or in modulating proteinase activity[127]. Matricellular proteins have the capacity to moderate strong cell adhesion, without disrupting cell morphology, and thereby possess 'de-adhesive' activity[125]. Changes in cardiomyocyte adhesive strength in the cardiac ECM may provide the cells with enhanced migratory phenotype while maintaining a spread morphology that is also important in preventing the onset of anoikis in anchorage-dependent cells[125,127].

Without subserving a direct structural role in the ECM, matricellular proteins act by binding matrix proteins, cell surface receptors or secreted molecules including cytokines. These interactions regulate cell function along with cell-matrix interactions and may contribute to their role in wound healing and tissue remodeling. Osteopontin in particular, possesses the capacity to interact with multiple cell surface receptors and has even been coined as a 'survival molecule' through its ability to stimulate antiapoptotic signalling pathways[90]. These qualities contribute to the exciting potential of matricellular proteins as tools for regenerative therapies, and emphasize the importance of directing more attention to the development novel strategies to take advantage of their unique properties.

1.2.6 Osteopontin

Osteopontin is a multifunctional matricellular protein that mediates interactions between cells and their ECM via multiple cell surface receptors. These binding sites include integrin heterodimers $\alpha_v(\beta_1, \beta_3, \text{ or } \beta_5)$ and $\beta_1(\alpha_4, \alpha_5, \alpha_8 \text{ or } \alpha_9)$ which bind osteopontin in its native form, as well as $\alpha_9\beta_1$ which binds to the cryptic SVVYGLR sequence once exposed by thrombin cleavage[130]. Additionally, osteopontin exhibits paracrine and autocrine signaling by serving as a ligand for variant forms of CD44 (v6 and/or v7)[131]. Varying levels of post-translational modifications such as phosphorylation, glycosylation and sulphation of the nascent protein result in different functional forms of osteopontin, localized in various tissues. Although secreted osteopontin deposits into the matrix of mineralized connective tissue, it is also present in biological fluids including blood[132].

Osteopontin displays both reparative and pathogenic potential. Reparative potential of osteopontin has been demonstrated in arterial injury rabbit models, in which local delivery of

recombinant osteopontin at sites of injury enhanced healing and reduced stenosis[133]. Additionally, osteopontin can promote endothelial cell adhesion and survival due to its multiple integrin binding sites, primarily through $\alpha_v\beta_3$ binding and activation of NF-κB signaling[134,135,136]. However, these integrin binding sites also mediate the pro- and anti-inflammatory effects induced by osteopontin, which regulate inflammatory cell adhesion, migration, cytokine secretion, differentiation state along with macrophage recruitment[90]. Pro-inflammatory effects of osteopontin have been reported in part by acting as a T-helper 1 cytokine, subsequently exacerbating inflammation in chronic inflammatory diseases, such as atherosclerosis[137]. In contrast, anti-inflammatory effects demonstrated by osteopontin correlate to its ability to inhibit inducible nitric oxide (NO) synthase induction[138]. In vitro studies have demonstrated reduced NO synthesis as a result of OPN-mediated downregulation of inducible NO synthase (iNOS) in macrophages and kidney tubule epithelial cells [138,139].

As mentioned earlier under Section 1.2.4, osteopontin is also known to be a substrate for TG2[100,120,140-142]. Higashikawa et al. (2007) recently demonstrated that osteopontin polymerized by TG2 promotes greater cell adhesion, spreading, migration and focal contact formation than monomeric osteopontin[101]. The combined properties of TG2's capacity to stabilize matrices and the ability of osteopontin (monomeric and polymeric) to promote cell survival and adhesion by engaging multiple integrin receptors, in conjunction with its reparative potential, are the very properties that make these matrix proteins ideal candidates to enhance cell therapies.

1.3 Microencapsulation

Binding of cell surface integrins to immobilized ligands leads to enhanced cell survival mediated through appropriate intracellular signaling. To mimic these responses in suspended cells, I have employed a single cell encapsulation strategy to immobilize matricellular proteins within a hydrogel scaffold surrounding individual cells. MSC encapsulation can be utilized as a means to deliver a suspension of individual encapsulated stem cells to the target tissue by intravascular injection. Encapsulated stem cells lodge within the microvasculature,

migrate from the capsule and engraft in the surrounding tissue, while sustaining the capacity of MSCs to self-renew, differentiate into numerous phenotypes, and migrate to sites of injury. Unlike previous *in vivo* encapsulation techniques, this strategy does not primarily focus on isolating allogeneic or xenogeneic cells from the host's immune system[143]. Instead, it provides a temporary microenvironment to enhance cell survival while allowing the exchange of nutrients and cell products. This capsule can be engineered to promote eventual migration of the individual cell to the surrounding tissue, a prerequisite for cell engraftment.

Hydrogels are the material of choice for cell encpsulation. Both synthetic and naturally derived hydrogels are used as a tool for cell immobilization, cell transplantation and tissue engineering. Lim and Sun's (1980) encapsulation platform consisting of calcium alginate hydrogels stabilized with a polylysine coating is the most commonly used model for capsules[144,145]. These polymers are non-degradable and thereby encapsulate transplanted cells for a prolonged period and encapsulate hundreds to thousands of cells in each microcapsule. In our method, agarose was selected as the capsule medium as 1) it provides a temporary microenvironment; 2) it does not interfere with cell signaling effects since it does not alter or bind the cell membrane; 3) forms a permeable structure allowing the passage of therapeutic and waste products as well as growth factors[146]; and 4) produces small microdrops that can be easily processed with a flow cytometer[146,147].

Microcapsules comprised of hydrogel alone however do not provide integrin binding partners that mediate cell-matrix interactions, and thus do not mimic a microenvironment that can suppress the onset of anoikis. Supplementing microcapsules with adhesion molecule ligands such as fibronectin (FN) and fibrinogen (FG) has been shown to promote MSC survival in suspension culture. FN is a ubiquitous adhesion molecule present as a polymeric fibrillar network in the ECM and as soluble protomers in plasma and other body fluids. Each protomer consists of three types of homologous structural repeats named FN type I, type II and type III repeats linked via disulfide bridges. Alternative splicing of the FN gene generates over 20 isoforms of the multifunctional glycoprotein. As a product of multiple cell types, FN serves an important role in cell adhesive and migratory functions. Similar to FN, FG is a large, complex, fibrous glycoprotein comprised of 3 subunits of polypeptide chains,

linked together by disulfide bonds. FG, normally found as a soluble macromolecule in blood plasma is an integral participant in the coagulation cascade[148]. As a multifunctional protein, FG also plays an important role in hemostasis, wound healing, inflammation angiogenesis and as a cell adhesion protein[148]. Both FG and/or its derivative fibrin can bind numerous proteins including FN, albumin, thrombospondin, von Willebrand factor, fibulin, fibroblast growth factor-2, vascular endothelial growth factor along with interleukin-1[148]. FG and fibrin binding to many of these specific molecules alters the structure and adhesive properties of clots important in the coagulation cascade and wound healing[148]. Both FG and FN contain integrin binding sites, primarily interacting with αvβ3 and α5β1 respectively[149-154]. Interactions with these cell surface receptors have been shown to play a prominent role in supporting viability of encapsulated MSCs maintained in suspension cultures[146].

Previous studies have shown that engineering microcapsules with β1 and β3 integrin binding partners (FN and FG, respectively) will engage cell surface receptors and enhance cell survival, as well as promote migration of cells out of capsules and engraftment into the surrounding tissue[146]. Basic *in vitro* studies on MSCs maintained in agarose capsules supplemented with FN and FG demonstrated nearly two-fold enhanced cell migration out of capsules and approximately 10% increased MSC survival relative to MSCs in agarose capsules with no matrix protein supplementation[146]. Recent data identified β1 integrins as a distinctive pathway for MSC migration and engraftment within ischemic myocardium[155]. Pulmonary injury animal models have shown 5-fold greater retention and survival of encapsulated cells 3 weeks post delivery in elastase-damaged rat lungs[146]. These results implicate the importance of the ECM in enhancing cell survival, migration and engraftment.

Thus, cell microencapsulation using agarose based microcapsules supplemented with integrin binding partners is a promising cell delivery strategy for promoting cell viability and targeted cellular engraftment. In order to optimize this technique, specific protein supplementation and the subsequent effects need to be carefully considered. Matricellular protein supplementation in microcapsules may be ideally suited to enhance this system as these proteins can function to modulate cell-matrix interactions and thereby regulate cell functions to promote cell survival. Denhardt et al. have suggested that osteopontin, through

its ability to interact with cell surface receptors mediated by multiple integrin binding sites, stimulates anti-apoptotic signaling pathways and thereby exerts a role as a survival molecule[90]. In addition, TG2 catalyzed polymerization results in enhanced biological activity of osteopontin[101] that may further strengthen MSC-matrix interactions. This constellation of properties in conjunction with the direct reparative activities of osteopontin discussed earlier under Section 1.2.5 suggest that osteopontin supplementation of hyodrogel microcapsules may provide an ideal strategy to enhance MSC cell therapy. Although, osteopontin demonstrates both pro- and anti-inflammatory effects[90], these qualities can be controlled with low levels of protein supplementation and administering supplemented microcapsules in a carefully managed fashion. Therefore, supplementing microcapsules with optimal levels of matricellular proteins will potentially engage cell surface receptors and thereby up-regulate cell survival signalling pathways and in turn enhance the therapeutic potential of MSC-based therapy.

1.4 Thesis Rationale & Objectives

Low cell survival and organ-specific cell retention have reduced the success of cell-based therapies. To overcome these limitations, a matrix-supplemented microencapsulation technique has been developed to promote cell engraftment in target tissues after intravascular injection, while sustaining high cell viability. Microcapsules, prepared with agarose gel supplemented with matrix proteins (FN/FG), have been shown to enhance survival and pulmonary retention of syngeneic rat MSCs[146]. Although current FN/FG supplemented capsules offer significant potential, further optimization of microcapsules to enhance cell viability and engraftment is needed to maximize their therapeutic potential. Thus, this thesis will explore the effects of incorporation of a matricellular protein (osteopontin) and an ECM cross-linking enzyme (TG2) on the ability of microcapsulation to enhance cell survival and activity. Accordingly, the overall objectives of my research project are as follows,

1) To compare the effects of osteopontin, and its different morphological forms, on MSC adhesion, morphology, cytoskeletal arrangement, focal contact formation and pluripotency.
2) To examine the effects of osteopontin supplementation on encapsulated MSC viability maintained in suspension culture.

Hypotheses

1) Osteopontin will interact with MSCs such that it will augment cell adhesion and cell spreading.
2) Osteopontin polymerized by TG2 will serve as a more potent matrix protein relative to monomeric osteopontin, promoting greater MSC adhesion, spreading and focal contact sites.
3) Supplementation of microcapsules containing FN and FG with polymeric osteopontin and TG2 will prevent MSC anoikis while maintained in suspension cultures.
4) Polymeric osteopontin and TG2 will act synergistically with the other microcapsule components, FN and FG, to protect MSCs from anoikis.

2.0 Description of the Methodology

Methodologies were tailored to appropriately test the above respective hypotheses and fulfill the project objectives and specific aims. Due to the unique nature of the projects outlined in this thesis, a number of more standard methods were tailored to the respective assays, while a selective group of original methodologies were uniquely developed to meet the customary needs of the studies in question. These particular methods were therefore adapted accordingly and validated prior to implementation.

2.1 Cells and Cell Culture

Passage 1 cryopreserved human multipotent stromal cells (hMSCs) were obtained from Tulane Centre for Gene Therapy (New Orleans; LA) in frozen vials containing approximately one million cells. The Tulane Centre for Gene Therapy isolated, expanded and characterized the hMSCs and provided detailed protocols that were followed for cell culturing purposes[156]. Primary hMSCs were cultured at 1000cells/cm^2 in T-75cm^2 tissue culture flasks (Corning Inc. Life Sciences, Lowell, MA) maintained at 37°C in a humidified 5% CO_2 environment. Cells were cultured with Complete Culture Medium (CCM) composed of Alpha Minimum Essential Medium (α-MEM; Invitrogen Canada Inc., Burlington; Canada) supplemented with 16.5% fetal bovine serum (FBS; Atlanta Biologicals Inc., Lawrenceville, GA), 1% L-glutamine (Invitrogen Canada Inc., Burlington; Canada), and 1% penicillin-streptomycin (Invitrogen Canada Inc., Burlington; Canada). All experiments were conducted on cells at passage 3 – 5 with media changes every 3 days.

In order to harvest cells, CCM was first removed by vacuum aspiration and cells were washed once with Phosphate Buffered Saline (PBS; Invitrogen Canada Inc., Burlington; Canada). Cells were treated with 0.25% Trypsin/EDTA (Invitrogen Canada Inc., Burlington; Canada) for 1-3 minutes before the trypsin was neutralized with an equal volume of CCM. The cell suspension was extracted into a 15mL conical tube, and any remaining cells were collected by washing flasks with PBS. Centrifugation of the cell suspension at 450g for 10

minutes generated a pellet of intact cells at the bottom of the conical tube. Cells were resuspended in 1mL of CCM and counted using trypan blue and a haemocytometer.

2.2 Osteopontin Polymerization by Transglutaminase 2

Osteopontin was polymerized as previously described[101]. Recombinant human osteopontin (R&D Systems, Minneapolis, MN) was incubated with recombinant human transglutaminase 2 (TG2; R&D Systems, Minneapolis, MN) at 50µg/mL and 100µg/mL respectively in a reaction mixture solution composed of 50mM Tris-HCl (pH 7.5), 5mM $CaCl_2$ and 1mM DTT for 2 hours at 37°C and 5% CO_2. TG2 was not removed from the final preparation. The formation of high molecular weight osteopontin polymers was confirmed by Western blotting.

2.3 Western Blot

Following osteopontin polymerization, 0.5µg of the resultant proteins were loaded into each lane of a 8-12% Tris-Glycine gel (Invitrogen Canada Inc., Burlington; Canada). Proteins were transferred onto a PVDF membrane following a one and half hour transfer at 100V on ice. Unoccupied protein binding sites on the membrane were blocked by placing the membrane in a blocking buffer (3% Bovine Serum Albumin in Tris-Tween Buffered Saline) and then probed with an anti-osteopontin monoclonal antibody (1µg/mL; R & D Systems, Minneapolis, MN; Clone: 223126) followed by a goat anti-mouse HRP conjugated secondary antibody (1:2000; R & D Systems, Minneapolis, MN).

2.4 Cell Adhesion Assays

Prior to the establishment of cell adhesion assay protocols, the appropriate cell density of cells/well and the respective adhesion time frame were investigated by examining numerous cell densities and adhesion times.

Flat bottom 96-well ELISA plates were coated with 100µL of protein per well. Plates were incubated overnight at room temperature. Unbound protein was removed by gentle aspiration and washing wells three times with PBS. Plates were then blocked with 200µL per well of 0.5% Bovine Serum Albumin (BSA; Sigma-Aldrich; Oakville, Canada) in PBS at room

temperature for 1 hour. Block solution was removed at end of the incubation by gentle aspiration. Plates were then incubated for 10 minutes at 37°C and 5% CO_2. In order to achieve even dispersion of cells, 50µL of serum-free CCM was added to each well, followed by the addition of 5000 cells in 50µL of serum-free CCM along the wall of each well. The cell suspension and CCM mixture were pipetted up and down 2-3 times to achieve even dispersion of cells. This particular cell density was selected based on the high cell number per field which does not result in cell-cell interactions. Prevention of cell-cell contact is important such that it does not interfere with assessment of cell-matrix associations. Cells were then allowed to adhere for 2 hours at 37°C and 5% CO_2. The adhesion time selected was also examined with supplementary experiments testing cell adhesion in time frames of 1, 2 and 4 hours. As depicted in Figure 2.1, cells allowed to adhere to osteopontin (monomeric or polymeric) and TG2 for either 1, 2 or 4 hours demonstrated a peak in cell adhesion at 2 hours. Thus, 2 hour adhesion time frames were used for all adhesion assays. Non-adherent cells were removed at the end of the incubation by inverting the plate onto an absorbent pad. Wells were then washed two times with serum-free CCM inserted along the wall of each well. Adherent cells were then fixed by incubating them with 100µL of cold 2% paraformaldehyde for 10 minutes at room temperature. Fixative solution was removed by inverting the plate onto an absorbent pad and washing two times with cold PBS. Wells were then carefully filled to the top with PBS inserted along the top wall of the well and covered with glass coverslips to enhance image quality. Adherent cells were assessed by counting cells in 5 fields using phase contrast microscopy at 10X (Nikon Eclipse TS100 microscope). Assays were completed three times in triplicate.

2.5 Cytoskeletal and Focal Contact Staining of Adherent Cells

8-well chamber glass slides were coated with 200µL of either 0.1% BSA in PBS, monomeric osteopontin (20µg/mL), polymeric osteopontin preparation (20µg/mL) or transglutaminase 2 (40µg/mL)and incubated overnight at room temperature. Unbound protein was removed by gentle aspiration, and wells were washed three times with PBS. Wells were then blocked with 400µL of 0.5% BSA in PBS for 1 hour at room temperature and incubated at 37°C and 5% CO_2 for 10 minutes. Prior to the addition of cells 100µL of serum-free CCM

was added to each well, followed by the addition of $1.1x10^4$ cells in 200µL of serum-free CCM. The mixture was pipetted up and down two times to achieve even distribution of cells. Cells were allowed to adhere for 2 hours at 37°C and 5% CO_2. Non-adherent cells were removed at the end of the incubation by inverting the plate onto an absorbent pad. Wells were then washed two times with serum-free CCM inserted along the wall of each well. Adherent cells were then fixed by incubating them with 120µL of cold 2% paraformaldehyde for 10 minutes at room temperature. Fixative solution was removed by inverting the plate onto an absorbent pad and washing two times with cold PBS. Adherent cells were permeabilized with 0.1% Triton-X in PBS (120µL/well) for 3 minutes, followed by two 3 minute washes with PBS. Non-specific binding was blocked by incubating wells with a Vectastain Elite ABC Kit blocking serum (120µL/well; Vectastain Elite ABC Kit, Vector Laboratories Inc.; Burlington, Canada) for 20 minutes at room temperature. Focal contact sites were probed with a monoclonal anti-vincullin antibody (Sigma-Aldrich; Oakville, Canada; Clone: hVIN-1) at 1:500 in PBS for 1 hour at 37°C. No primary antibody and irrelevant isotype antibodies were also used as negative controls. Antibody solution was removed by inverting plate and conducting three 5 minute washes with PBS. Anti-vincullin primary antibodies were then probed with a biotinylated anti-mouse IgG secondary antibody (Vectastain Elite ABC Kit) for 30 minutes at room temperature and subsequently washed three times with PBS for 5 minutes during each wash. Secondary antibody sites were then tagged with Streptavidin Alexa Fluor 488 (Molecular Probes Invitrogen Canada Inc., Burlington; Canada) at a final concentration of 1µg/mL, for 1 hour at room temperature, followed by two 5 minute washes in PBS. Cytoskeletal network (F-actin) and nuclear staining were conducted with a 20 minute incubation at room temperature with Alexa 546 conjugated phalloidin (13µL/mL; Molecular Probes Invitrogen Canada Inc., Burlington; Canada) and ToPro3 (1µL/mL; Molecular Probes Invitrogen Canada Inc., Burlington; Canada) staining. Samples were washed twice with PBS for 5 minutes before being mounted in mowiol and stored overnight at 4°C. Samples were assessed with confocal microscopy (Nikon Eclipse E800 confocal microscope). Assays were completed at least twice on separate days, each time with the negative, positive and isotype controls described above.

2.6 Quantitative Immunohistochemistry

Confocal microscopy images of adherent cells stained for vinculin in the presence of 0.1% BSA in PBS, monomeric osteopontin (20μg/mL), polymeric osteopontin and TG2 (20μg/mL and 40μg/mL, respectively) or TG2 alone (40μg/mL) were prepared and captured as described above. Cells were then assessed by manually tracing the outline of each cell and establishing a baseline pixel intensity threshold value for all images examined using image analysis software (ImageJ 1.39o)[157]. This value was determined based on the ImageJ automatic thresholding function which distinguishes objects from image background by computing a test threshold value based on the average of pixels above and below the test threshold [*threshold = (average background + average objects)/2*]. The test threshold value is then incremented and the process is repeated until the test threshold value is larger than the composite average. Image analysis software was then automated to quantify the number of focal contact sites (defined as objects) based the threshold value of an image of adherent cells with well defined focal contact sites in the presence of polymeric osteopontin. Experiments were completed three times with 20 cells examined in each experiment.

2.7 Cell Morphometry

Following multimerization of osteopontin by transglutaminase 2, flat bottom 96-well plates were coated with 100μL/well of protein (0.1% BSA in PBS, osteopontin: 20μg/mL; TG2: 40μg/mL). Plates were incubated overnight at room temperature after which unbound protein was then removed by gentle aspiration and washing wells three times with PBS. Wells were then blocked with 200μL of 0.5% BSA in PBS at room temperature for 1 hour. Block solution was removed at the end of the incubation by gentle aspiration and plates were then warmed by incubating for 10 minutes at 37°C and 5% CO_2. In order to achieve even dispersion of cells, 50μL of serum-free media was added to each well, followed by the addition of 5000 cells in 50μL of serum-free media along the wall of each well. The cell suspension and media mixture were pipetted up and down two to three times to achieve even dispersion of cells. Cells were then allowed to adhere for 2 hours at 37°C and 5% CO_2. In order to examine samples using microscopy, wells were carefully filled to the top with serum-free media inserted along the wall of each well and covered with glass coverslips to enhance image quality. Phase contrast images were captured on a Nikon Eclipse TS100

microscope and analyzed via image analysis software. Cells were assessed by tracing the outline of each cell. The area (μm^2) and circularity ($circularity = 4\pi\left(\frac{area}{perimeter^2}\right)$) were calculated using ImageJ image analysis software. A circularity value of 1.0 is characteristic of a perfect circle, and as the value approaches 0.0, it is depictive of an increasingly elongated shape deviating from a circular shape. Morphometry assays were repeated six times with 250 cells examined in each experiment.

2.8 MSC Differentiation

8-well chamber well glass slides were coated with 200µL of either monomeric osteopontin (20µg/mL), polymeric osteopontin preparation with TG2 (20µg/mL osteopontin and 40µg/mL TG2) or TG2 alone (40µg/mL) and incubated overnight at room temperature. Unbound protein was removed by gentle aspiration, and wells were washed three times with PBS. Wells were then blocked with 400µL of 0.5% BSA in PBS for 1 hour at room temperature and incubated at 37°C and 5% CO_2 for 10 minutes. Prior to the addition of cells 100µL of serum-free media was added to each well, followed by the addition of $1.1x10^4$ cells/cm^2 in 100µL of serum-free media. The mixture was pipetted up and down two times to achieve even distribution of cells. Cells were allowed to grow to 50-70% confluency (in 1 day) after which they were cultured in the respective supplemented media (adipogenic and osteogenic supplements in CCM, and chondrogenic supplement in CCM prepared with DMEM/F12; Human Mesenchymal Stem Cell Functional Identification Kit; R & D Systems, Minneapolis, MN) for 21 days. Cells cultured in standard CCM with no supplements were also maintained for 21 days as undifferentiated controls. Media changes were conducted every three days, with fresh supplemented media prepared immediately before. At the commencement of the 21 day culture period, media was removed from wells by gentle aspiration. Wells were then washed two times with PBS inserted along the wall of each well. Cells were then fixed by incubating them with 200µL of 4% paraformaldehyde for 20 minutes at room temperature. Fixative solution was removed by inverting the plate onto an absorbent pad and washing adipogenic and osteogenic cells three times with 1% BSA in PBS for 5 minutes, and washing chondrogenic cells two times with PBS for 5 minutes. Cells were permeabilized and non-specific binding was blocked by incubating cells with 0.3% Triton-X-

100, 1% BSA and 10% normal donkey serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA) in PBS at room temperature for 45 minutes. Lipid vacuoles of adipocytes were probed with a polyclonal anti-Fatty Acid Protein-4 (FABP-4; Human Mesenchymal Stem Cell Functional Identification Kit; R & D Systems) antibody, while secreted extracellular matrix molecules osteocalcin (osteogenic) and aggrecan (chondrogenic) were probed with monoclonal anti-osteocalcin and polyclonal anti-aggrecan antibodies (Human Mesenchymal Stem Cell Functional Identification Kit; R & D Systems), respectively, at 10μg/mL in 1% BSA and 10% normal donkey serum in PBS overnight at 4°C. No primary antibody and irrelevant isotype antibodies were also used as negative controls. Wells were then washed 3 times with PBS for 5 minutes, after which primary antibodies were probed with either Rhodamine red-conjugated donkey anti-goat secondary antibody (adipogenic and chondrogenic; Jackson Immunoresearch Laboratories Inc.) or Rhodamine red-conjugated donkey anti-mouse secondary antibody (osteogenic; Jackson Immunoresearch Laboratories Inc.) in the dark for 1 hour at room temperature. Samples were washed three times with PBS for 5 minutes before being mounted in mowiol, stored overnight at 4°C and finally assessed with fluorescent microscopy (Nikon Eclipse E800 fluorescent microscope).

In order to first confirm pluripotency of MSCs, supplemental assays were employed where MSCs were promoted to differentiate without the presence of adhesion molecule ligands (0.1% BSA in PBS; Figure 2.2A). Differentiation of the cells to the respective lineages confirmed that these cells are pluripotent and can thereby be defined as MSCs. In addition, MSCs were also cultured in the presence of the above mentioned protein coatings in standard cell culture media to confirm that each of the respective protein coated surfaces does not independently induce differentiation of MSCs (Figure 2.2B). All MSC differentiation assays were completed in duplicate with the negative, positive and isotype controls described.

2.9 Removal of Low Molecular Weight Proteins from Polymeric Preparation

Following osteopontin multimerization, samples were filtered with the Microcon Centrifugal Filter device (Millipore Corporation, Bedford, MA) with a molecular weight cut-off of 100kDa. Low molecular weight proteins would pass through the device membrane by spinning samples at 3000g for 3 minutes at room temperature. High molecular weight

proteins were recovered from the device by inverting the membrane into a new eppendorf tube and spinning at 1000g for 3 minutes at room temperature. These filtration and recovery steps were conducted a total of three times. Removal of low molecular weight proteins was then confirmed by western blotting and probing with a monoclonal anti-osteopontin antibody.

Following removal of residual TG2 and osteopontin monomers from multimerized samples by centrifugal filtration, total osteopontin remaining in recovered samples was measured by a Bradford protein assay of polymeric osteopontin samples.

2.10 Microencapsulation

Microcapsules were prepared as previously described[158]. Type IX-A ultra-low gelling point agarose (Sigma-Aldrich; Oakville, Canada) in PBS was supplemented with one of four combinations of matrix proteins; a standard capsule supplementation composed of 80μg/mL FN (Sigma-Aldrich; Oakville, Canada) and 800μg/mL FG (Sigma-Aldrich; Oakville, Canada), or the standard capsule preparation with further supplementation with either monomeric osteopontin (20μg/mL), polymeric osteopontin and TG2 (20μg/mL and 40μg/mL respectively) or TG2 (40μg/mL). Approximately 1.6 x 10^6 cells/1 mL of capsule preparation was resuspended in Hank's balanced salt solution (HBSS; Invitrogen Canada Inc.; Burlington, Canada) and then mixed with the supplemented agarose mixture in PBS. The mixture was further supplemented with 40uL 10% Pluronic F-68/1mL of the capsule preparation and finally emulsified in a drop-wise manner into dimethylpolysiloxane (DMPS) oil generating individual capsules. The mixture was placed on an ice bath for 10 minutes, promoting gelation of agarose droplets and subsequently split into two 15mL conical vial tubes where HBSS was added to each tube and then centrifuged at 652g for 10 minutes. The aqueous phase containing the encapsulated cells was collected and washed once with 0.5% BSA in HBSS and centrifuged at 453g for 5 minutes at room temperature. The final pellet was re-suspended in serum-free cell culture media and filtered through a 70μm cell filter.

2.11 Generating Suspension Cultures – PolyHEMA Coatings

Suspension culture conditions were first prepared by coating 6-well (flow cytometry assays) or 384-well (fluorimetric assays) plates with poly (2-hydroxyethyl methacrylate) [poly-Hema]. A 12% poly-Hema solution in 90% ethanol was prepared in a 15mL conical tube, shaken vigorously and incubated overnight at 37°C to enhance solubility. Any remaining undissolved material was solubilized by centrifuging at 1258g for 30 minutes. 6-well or 384-well plates were then coated with 0.1mL/cm^2 of the poly-Hema solution and incubated at 37°C for 40-48 hours to allow the ethanol to evaporate. Plates were finally washed twice for 5 minutes with PBS before use.

2.12 Apoptosis Assay – Annexin V & Propidium Iodide Staining

Prior to the establishment of a protocol for Annexin V and Propidium Iodide staining detection by flow cytometry, supplemental experiments were conducted to evaluate the encapsulated cell population using flow cytometry. In order to examine this particular population, original flow cytometry protocols were developed to eliminate cell/microcapsule debris as well as non-encapsulated cells (within the microcapsule preparations) from the population of encapsulated cells. Cells were labeled with CMTMR live cell tracker (2.5µM for 30 minutes at 37ºC and 5% CO_2). Cells were then encapsulated in the FN/FG microcapsule formulation described earlier, consisting of 80µg/mL of FN and 800µg/mL of FG. The FG content in these microcapsule preparations consisted of approximately 40µg of Oregon-green conjugated FG/1mL of capsule preparation and the remaining FG content was in the form of non-fluorescently conjugated FG generating a total FG content of 800µg/mL. Fluorescently labeled encapsulated cells were then assessed by flow cytometry collecting in the FL1 and FL2 channels (525nm and 575nm emission detectors, respectively). The population of double positive (CMTMR and Oregon-green positive) events were gated and subsequently isolated based on size and granularity (Figure 2.3). This gate was used in subsequent flow cytometry assays to evaluate the encapsulated cell population from microcapsule preparations.

Once encapsulated cells were prepared, 8.0 x 10^4 cells in 2 mL of serum-free media were inserted into appropriate wells of a 6-well plate precoated with poly-Hema (as described above) or non-coated wells (for non-encapsulated adherent culture cells). Cells were incubated at 37°C and 5% CO_2 for 24 hours before being harvested by trypsinization (if not encapsulated, ie. adherent culture) or simply by collection and spinning at room temperature for 5 minutes at 450g (if encapsulated). Samples were washed once with PBS, and subsequently resuspended in staining solution composed of 2μL Annexin V and 2μL Propidium Iodide in 100μL staining buffer (Annexin-V-FLUOS Staining Kit; Roche Diagnostics; Mannheim, Germany). Samples were incubated at room temperature for 15 minutes in the dark, washed once with cold PBS, resuspended in 500μL staining buffer and analyzed by flow cytometry using the Beckman Coulter Cytomics FC500 (488nm laser excitation) collecting in the FL1 and FL3 channels (525nm and 610nm emission detectors, respectively). Experiments were repeated three times, where each time 1.0x10^4 events were examined.

2.13 Apoptosis Assay – Caspase Activity

Cells were encapsulated as described above and once encapsulated 10^4 cells in 25μL of serum-free media were inserted into appropriate wells of a 384-well plate precoated with poly-Hema (as described above). Cells were incubated at 37°C and 5% CO_2 for 3 hours before 25μL of DEVD-R110 caspase substrate (Homogeneous Caspase Assay; Roche Diagnostics; Mannheim, Germany) was added to each well and incubated for 90 minutes at 37°C and 5% CO_2. Fluorimetric measurement of free R110 concentration was then conducted with an excitation filter at 485nm and an emission filter at 518nm using the Fluoroskan Ascent FL Thermo LabSystems. The resulting fluorochrome is proportional to the concentration of activated caspases. Assays were completed in triplicate.

2.14 Osteopontin Staining in Microcapsules

Standard microcapsules with and without FN (80μg/mL) and FG (800μg/mL) were supplemented with either monomeric osteopontin (20μg/mL) or polymeric osteopontin and TG2 (20μg/mL and 40μg/mL, respectively). Microcapsules were prepared as described

above and resuspended in 300μL of the Vectastain Elite ABC Kit blocking serum for 20 minutes at room temperature. Blocking solution was removed by centrifuging capsules at 1500rpm for 3 minutes and subsequently removing the supernatant. Osteopontin immobilized in the capsules was probed with a monoclonal anti-osteopontin antibody (1μg/mL) in PBS for 1 hour at 37°C. No primary antibody and irrelevant isotype antibodies were also used as negative controls. Antibody solution was removed by centrifuging capsules at 1500rpm for 3 minutes, removing the supernatant and conducting three 5 minute washes with PBS. Anti-osteopontin primary antibodies were then probed with a biotinylated anti-mouse IgG secondary antibody (Vectastain Elite ABC Kit) for 30 minutes at room temperature and subsequently washed three times with PBS for 5 minutes during each wash. Secondary antibody sites were then tagged with Streptavidin Alexa Fluor 488 at a final concentration of 1μg/mL, for 1 hour at room temperature, followed by two 5 minute washes in PBS. Samples were stored overnight at 4°C and inserted onto glass coverslips before being assessed with confocal microscopy (Leica TCS SL Confocal System).

2.15 Incorporating Fluorescently Tagged Fibrinogen into Microcapsules

Standard microcapsules containing FN (80μg/mL) and FG (800μg/mL) and further supplemented with either monomeric osteopontin (20μg/mL) or polymeric osteopontin and TG2 (20μg/mL and 40μg/mL, respectively) were prepared as described. The FG content in the microcapsule preparations consisted of approximately 40μg of Oregon-green conjugated FG/1mL of capsule preparation and the remaining FG content was in the form of non-fluorescently conjugated FG generating a total FG content of 800μg/mL. Once microcapsules were prepared, samples were inserted onto glass coverslips and assessed with confocal microscopy (Leica TCS SL Confocal System).

2.16 Statistical Analysis

All data is expressed as the mean of a sample group (of at least $n = 3$) ± standard error of the mean. Statistical significance was assessed with analysis of variance or student's t test where a p value < 0.05 was defined as statistically significant.

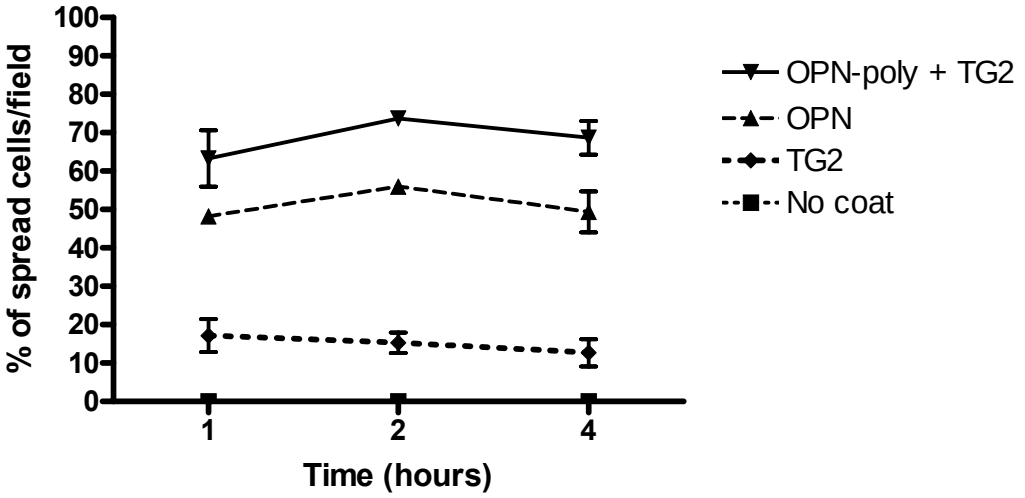

Figure 2.1: Time course of human MSC adhesion to monomeric and polymeric osteopontin as well as TG2 adsorbed to plastic. 5000 cells in 100µL of serum-free media were plated onto wells coated with PBS+0.1%BSA (no coat; ■), 20µg/mL of monomeric osteopontin (▲), 20µg/mL polymeric osteopontin and 40µg/mL TG2 (▼) or 40µg/mL TG2 alone (▪). Cells were allowed to adhere for 1, 2 or 4 hours. The percentage of cells exhibiting extensions and spreading was quantified in five fields. Each plot represents the mean (±SEM). [n=3, each experiment is done in triplicate samples]

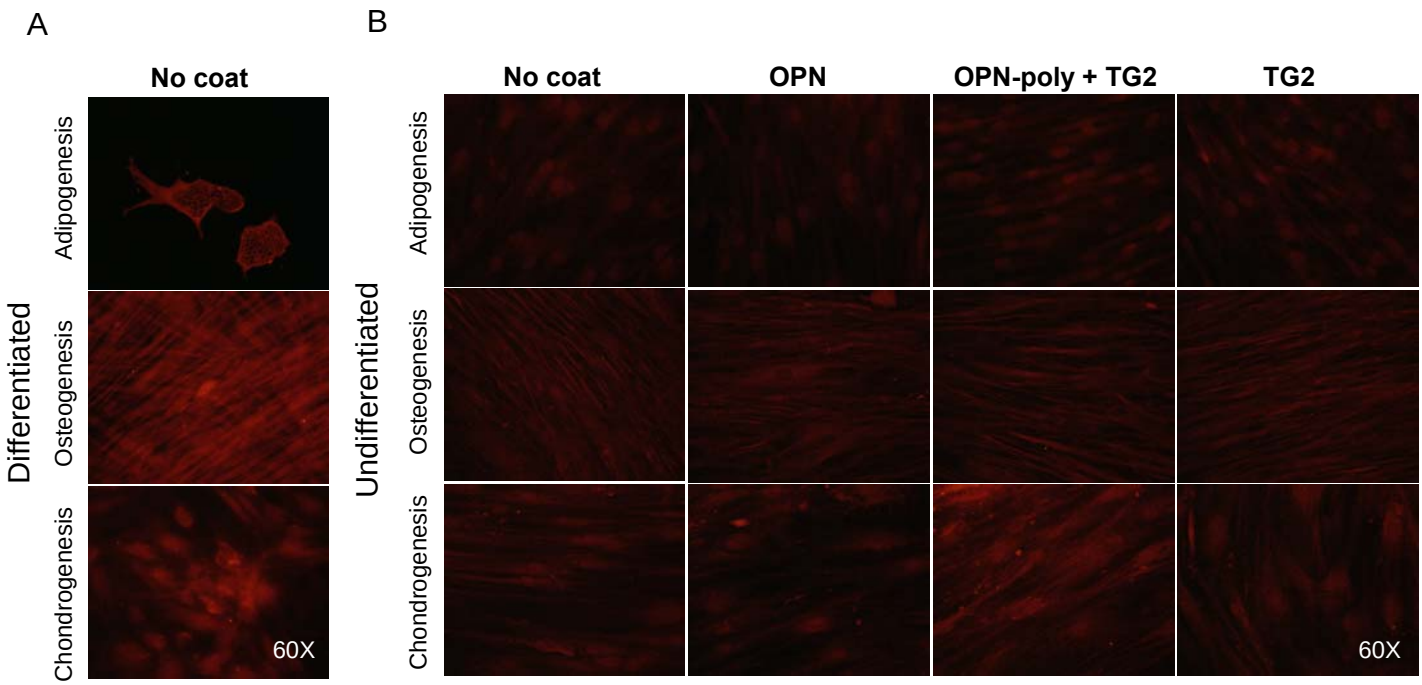

Figure 2.2: Pluripotency of human MSCs. 8-well chamber well glass slides coated with either 0.1% BSA in PBS (no coat; A & B), 20µg/mL monomeric osteopontin (B), 20µg/mL polymeric osteopontin and 40µg/mL TG2 (B) or 40µg/mL TG2 alone (B). $1.1x10^4$ cells/cm^2 were plated in each well and cultured in standard cell culture media (undifferentiated, B) or adipogenic, osteogenic or chondrogenic supplemented media (differentiated, B) for 21 days. Differentiated and undifferentiated cells were probed with polyclonal anti-Fatty Acid Binding Protein-4 antibody (adipocytes), monoclonal anti-osteocalcin antibody (osteocytes) or polyclonal anti-aggrecan antibody (chondrocytes) followed by a Rhodamine Red-conjugated donkey anti-goat or donkey anti-mouse secondary antibody. Samples were assessed with fluorescent microscopy.

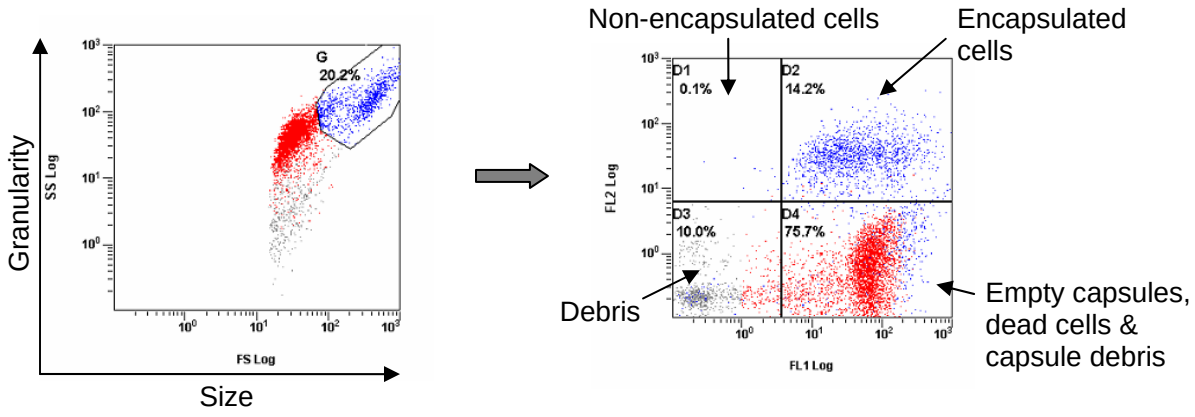

Figure 2.3: Examining encapsulated cells with flow cytometry. $1.6x10^6$ cells/1 mL of capsule preparation were in encapsulated in standard capsules supplemented with fibronectin (80µg/mL) and fibrinogen (800µg/mL) where Oregon-green conjugated fibrinogen was utilized. Cells were labeled with CMTMR live cell tracker prior to encapsulation. Fluorescently labeled cells and microcapsules were assessed by flow cytometry to characterize the encapsulated cell population according to size and granularity.

3.0 Project I: Effects of Monomeric and Polymeric Osteopontin on Multipotent Stromal Stem Cells

3.1 Study Aims and Rationale

The matricellular protein, osteopontin, exhibits promising reparative potential with the capacity to direct tissue repair and regeneration by regulating the behaviour of cells through interactions with multiple cell surface receptors[133-136]. Different morphological forms of this adhesive glycoprotein have been shown to alter its capacity to promote cell adhesion, spreading, migration and focal contact formation[101]. Crosslinking by TG2[100,120,140-142] results in the formation of osteopontin multimers that exhibit enhanced cell adhesive activity relative to monomeric osteopontin[101].

Matrix supplementation of extracellular microenvironments surrounding regenerative cells like MSCs with osteopontin may govern the subsequent fate and therapeutic potential of these cells. MSC differentiation and function are widely understood to be dictated, in part, by the surrounding microenvironment through complex cell-cell and cell-ECM interactions operating through ECM protein ligands and their respective cell surface receptors[159-164]. Therefore, in order to select candidates to optimize MSC-based therapy, it is essential to first elucidate the direct biological effects of the different morphological forms of osteopontin on MSCs.

Specific Hypotheses

1) Osteopontin will interact with MSCs by enhancing cell adhesion and cell spreading.
2) Osteopontin polymerized by TG2 will serve as a more potent matrix protein relative to monomeric osteopontin, inducing more marked changes in MSC morphology and promoting greater MSC adhesion and focal contact sites.

Project Aims:

To investigate the response of human MSCs to substrate immobilized osteopontin (monomeric and polymeric). The effects of monomeric osteopontin and TG2 polymerized osteopontin will be evaluated with quantitative assessment of cell adhesion, morphology and

focal contact formation, as well as qualitative examination of cytoskeletal networks and MSC pluripotency.

3.2 Methods

Recombinant human osteopontin was polymerized by a recombinant human TG2 mediated reaction, and this multimerization was confirmed by western blotting. Two dimensional assays where substrates were coated with respective proteins were employed to evaluate the responses of human MSCs, passages 3-5. Cell adhesion assays were completed three times in triplicate, while cell morphology assays were repeated six times with 250 cells evaluated in each experiment. Immunohistochemistry assays designed to examine cytoskeletal network, focal contact sites, and MSC differentiation were completed at least twice, each time with appropriate negative, positive and isotype controls in the presence of the different protein coated surfaces. Experiments detailing focal adhesion site quantification were repeated three times with 20 cells examined in each experiment. Detailed descriptions of corresponding methods are provided in Section 2.0.

3.3 Results

3.3.1 Transglutaminase 2 catalyzes osteopontin to form polymer

Transglutaminases catalyze protein multimerization by inducing the formation of isopeptide crosslinks from glutamine and lysine residues of their substrate proteins. Following osteopontin polymerization by TG2, the crosslinking enzyme was not removed from the final preparation. The formation of high molecular weight osteopontin polymers was then confirmed by Western blotting (Figure 3.1). Immunoblotting with a monoclonal anti-osteopontin antibody revealed a single band at approximately 60kDa corresponding to monomeric osteopontin, and a series of bands with molecular masses ranging from over 250kDa to less than 60kDa corresponding to the polymeric osteopontin preparation. Lack of immunoreactivity of TG2 with the anti-osteopontin antibody was shown in the lane corresponding to TG2 alone (Figure 3.1).

3.3.2 Enhanced cell adhesion to polymeric osteopontin

To compare the effects of monomeric osteopontin, polymeric osteopontin and TG2 on adhesion of human MSCs, I performed quantitative adhesion assays with varying concentrations of protein coated surfaces. Following multimerization of osteopontin by TG2, flat bottom 96-well plates were coated with 100μL/well of protein at one of five concentrations (monomeric or polymeric osteopontin: 1, 5, 10, 20 or 40μg/mL; TG2: 2, 10, 20, 40 or 80 μg/mL). 5000 cells were plated in each well and allowed to adhere for 2 hours at 37°C and 5% CO_2. Non-adherent cells were removed at the end of the incubation, and adherent cells were assessed by counting cells in 5 fields using phase contrast microscopy at 10X. As shown in Figure 3.2, MSCs adhered to all the protein coated surfaces in a dose-dependent manner. However, adhesion to the polymeric osteopontin preparation was significantly greater than monomeric osteopontin at higher concentrations (20 μg/mL: 47.1±7.86 vs. 72.7±14.7cells/field and 40 μg/mL: 44.3±10.0 vs. 84.5±10.7cells/field; monomeric vs. polymeric respectively; n=6, $p<0.05$).

3.3.3 Enhanced focal contact formation of MSCs on polymeric osteopontin

Morphological changes of MSCs in the presence of monomeric or polymeric osteopontin and TG2 was examined by the qualitative analysis of the cytoskeletal network and both qualitative and quantitative assessment of focal adhesion sites using immunostaining and fluorescent microscopy. $1.1x10^4$ cells/chamber were plated in 8-well glass slides that were precoated with 200μL of 0.1% BSA in PBS, monomeric osteopontin (20μg/mL), polymeric osteopontin preparation with TG2 (20μg/mL osteopontin and 40μg/mL TG2) or TG2 alone (40μg/mL). Non-adherent cells were removed at the end of the incubation and adherent cells were subjected to focal contact, cytoskeletal network and nuclear staining and finally examined with confocal fluorescent microscopy. Staining of adherent cells showed that MSCs exhibit well defined F-actin filaments and focal adhesion sites and a concentration of focal adhesion sites at the leading edges of adherent cells in the presence of polymeric osteopontin and TG2, (Figure 3.3). Cells cultured in the presence of monomeric osteopontin displayed less defined F-actin filament structure, and fewer distinct focal contact sites. Cells cultured without any adhesion molecules displayed a completely rounded morphology with no defined F-actin filament structure or focal contact formation, reflective on non-adherent cells. Quantification of focal adhesion sites using image analysis software revealed significantly greater number of focal contact sites (Figure 3.3B; 45.6±17.6 vs. 351.5±21.2 focal contact sites/cell; $p<0.001$; n=3; monomeric vs. polymeric respectively) and significantly greater number of focal contact sites per cell area (Figure 3.3C; 0.047±0.013 vs. 0.14±0.0064 focal contact sites/cell area; $p<0.001$; n=3; monomeric vs. polymeric respectively) in the presence of polymeric osteopontin. Interestingly, there was no difference in the number of focal contact sites and number of focal contact sites per cell area between cells in the presence of polymeric osteopontin and TG2 alone (351.5±21.2 vs. 362.5±42.0 focal contact sites/cell, respectively; n=3).

3.3.4 Enhanced cell spreading morphology of MSCs on polymeric oseopontin

The morphology of MSCs in the presence of monomeric or polymeric osteopontin and TG2 were further examined by quantitative analysis of the cell area and circularity. 5000 cells were plated in each well of flat bottom 96-well plates that were precoated with

100µL/well 0.1% BSA in PBS, monomeric osteopontin (20µg/mL), polymeric osteopontin preparation with TG2 (20µg/mL osteopontin and 40µg/mL TG2) or TG2 alone (40µg/mL). Cells were allowed to adhere for 2 hours at 37°C and 5% CO_2. Phase contrast images were then captured and analyzed via image analysis software on the basis of cell area and circularity. Quantitative analysis of MSC demonstrated distinct morphology of MSCs in the presence of polymeric osteopontin compared to monomeric osteopontin. Cells in the presence of polymeric osteopontin showed nearly 2-fold greater cell area (Figure 3.4A; $1.2x10^3 \pm 0.26x10^2 \mu m^2$ vs. $2.7x10^3 \pm 2.0x10^2 \mu m^2$; $p<0.001$; n=6) with morphology that was significantly less circular (Figure 3.4B; 0.51±0.039 vs. 0.30±0.024; $p<0.01$; n=6) than that in the presence of monomeric osteopontin. The morphology of cells in the presence of polymeric osteopontin was also significantly distinct from that of cells in the presence of TG2. Cells interacting with polymeric osteopontin displayed significantly greater cell area (Figure 3.4A; $2.7x10^3 \pm 2.0x10^2 \mu m^2$ vs. $1.7x10^3 \pm 0.94x10^2 \mu m^2$; $p<0.001$; n=6) with morphology that was significantly less circular (Figure 3.4B; 0.30±0.024 vs. 0.43±0.029; $p<0.05$; n=6) than that in the presence of TG2.

3.3.5 MSCs maintain pluripotency in the presence of monomeric and polymeric osteopontin

To determine whether MSCs maintain their pluripotency in the presence of monomeric and polymeric osteopontin, MSCs in the presence of 0.1% BSA in PBS, monomeric or polymeric osteopontin and TG2 were cultured in standard cell culture media (undifferentiated control) or adipogenic, osteogenic or chondrogenic supplemented media for 21 days, and finally examined with immunostaining and fluorescent microscopy. Lipid vacuoles of adipocytes were probed with a polyclonal anti-Fatty Acid Protein-4 (FABP-4) antibody, while secreted extracellular matrix molecules osteocalcin (osteogenic) and aggrecan (chondrogenic) were probed with monoclonal anti-osteocalcin and polyclonal anti-aggrecan antibodies, respectively. Primary antibodies were then probed with either Rhodamine red-conjugated donkey anti-goat secondary antibody (adipogenic and chondrogenic) or Rhodamine red-conjugated donkey anti-mouse secondary antibody (osteogenic) and finally assessed with fluorescent microscopy. Staining of differentiated cells cultured in the presence of no adhesion molecules, monomeric osteopontin, polymeric

osteopontin and TG2 or TG2 alone displayed large lipid vacuoles, and uniform osteocalcin and aggrecan formation characteristic of adipocytes, osteocytes and chondrocytes, respectively (Figure 3.5). Positive staining of FABP-4, osteocalcin and aggrecan in the corresponding samples suggests that MSCs maintain their pluripotency in long term culture conditions in the different microenvironments described.

3.3.6 Polymeric osteopontin induces MSC adhesion independently of TG2

To establish the specific effects of polymeric osteopontin, residual TG2 and monomeric osteopontin were removed from multimerized samples by size exclusion and assessed with quantitative adhesion studies. As shown in Figures 3.1 and 3.6A, monomeric osteopontin corresponds to a molecular weight of approximately 60kDa, while polymeric osteopontin ranges in sizes from above 250kDa to less than 60kDa. TG2 corresponds to a molecular weight of approximately 78kDa. Following osteopontin multimerization, samples were filtered with the Microcon Centrifugal Filtration device with a molecular weight cut-off of 100kDa. Removal of low molecular weight proteins was then confirmed by western blotting and probing with a monoclonal anti-osteopontin antibody. Western blotting of samples displayed a large reduction of low molecular weight osteopontin proteins as well as a slight reduction of high molecular weight polymers with increasing filtration steps, where a maximum of 3 filtration steps were performed (Figure 3.6A).

Quantitative adhesion studies were designed to compare the effects of polymeric osteopontin (independently of TG2) to those of monomeric osteopontin, polymeric osteopontin and TG2 as well as TG2 alone. Following removal of residual TG2 and osteopontin monomers from multimerized samples by centrifugal filtration, total osteopontin remaining in recovered samples was measured by a Bradford protein assay. Flat bottom 96-well plates were then coated with 100µL/well of 0.1% BSA in PBS; monomeric osteopontin (20µg/mL); polymeric osteopontin and TG2 (20µg/mL and 40µg/mL, respectively); or TG2 (40µg/mL). 5000 cells per well were plated and allowed to adhere for 2 hours at 37°C and 5% CO_2. Non-adherent cells were removed at the end of the incubation and adherent cells were assessed by counting cells in 5 fields using phase contrast microscopy. As shown in Figure 6B, polymeric osteopontin induced MSC adhesion independently of TG2. However,

the number of adherent cells in the presence of high molecular weight polymeric osteopontin was significantly less than that of polymeric ostepontin with TG2 (52.4±11.0 vs. 116.4±15.4cells/field; $p<0.05$; n=3), suggesting that TG2 may also play an important role in contributing to the effects induced by the polymeric ostepontin and TG2 complex on MSCs. In relation to monomeric osteopontin, there was no difference in the number of adherent cells in the presence of high molecular weight polymeric osteopontin (Figure 3.6B; 57.2±5.9 vs. 52.4±11.0cells/field; n=3; monomeric vs. polymeric, respectively).

3.4 Discussion

3.4.1 Osteopontin Polymerization

Transglutaminases mediate the formation of covalent bonds between free amine groups of substrate proteins, resulting in protein crosslinking[165,166]. Osteopontin multimerization by TG2 resulted in the formation of proteins ranging in size from 60 to over 250kDa. The formation of polymers with molecular masses varying in size, suggests that the multimers consist of a heterogeneous mixture of polymeric complexes. Heterogeneous multimer formation is consistent with observations from previous studies. Higashikawa et al. (2007) demonstrated a dose-dependent polymerization of recombinant osteopontin by TG2, generating polymers ranging in size from 30 to over 200 kDa[101]. Nascent monomeric osteopontin corresponds to a molecular weight of approximately 32 kDa, while post-translational modifications such as glycosylation generate osteopontin monomers of 60 to 65 kDa. Kaartinen et al. (1999) have also shown the formation of high molecular weight osteopontin polymers by TG2 crosslinking with osteopontin complexes ranging in molecular masses of 60 to over 250 kDa[120]. Insight into the development of heterogeneous complexes as a result of transglutaminase activity has also been gained by Factor XIIIa (TG) crosslinking of FN and FG to form dimers, which thereby crosslink to other hybrid dimers via their FG γ-chains and generate larger molecular weight oligomers[167]. Thus, TG2 crosslinking activity of osteopontin resulting in the formation of polymers ranging in size is consistent with previous studies and is a reflection of the development of a heterogeneous mixture of multimeric complexes.

3.4.2 Human MSC Adhesion and Phenotype in Response to Polymeric Osteopontin

Both polymeric and monomeric osteopontin were shown to induce adhesion of human MSCs in a dose dependent manner, while promoting morphological changes characteristic of adherent cells. However, relative to monomeric osteopontin, the polymeric form of this protein supports cell adhesion, elongation and focal contact formation more potently. In other words, the same relative concentration of polymeric osteopontin induces greater cell adhesion with enhanced cell elongation and focal contact formation than monomeric osteopontin. Although this is the first study examining the direct biological functions of

polymeric osteopontin on MSCs, Higashikawa et al. demonstrated in a recent study that osteopontin polymerized by TG2 promotes greater cell adhesion, spreading, migration and focal contact formation than monomeric osteopontin using a human colon carcinoma cell line and human umbilical cord endothelial cells[101]. Circular dichroism spectroscopy studies of osteopontin polymers have revealed an altered conformation of osteopontin to a more ordered structure[120]. Changes in conformation may serve to concentrate cell surface receptor ligand-binding sites and thereby enhance interactions with integrins by promoting integrin clustering, or may even expose cryptic epitopes recognized by β1 integrins[101]. Thus, the flexibility of osteopontin allowing changes in conformation upon polymerization by TG2 may be serving to amplify interactions with cell surface receptors and thereby induce more potent biological effects than monomeric osteopontin.

The crosslinking agent TG2 was not removed from polymeric osteopontin preparations and dramatic focal contact formation and cell spreading morphology were noted in the presence of polymeric osteopontin and TG2 complexes as well as TG2 alone. These observations suggest that TG2 may also be playing an important role as an adhesion molecule independently and in conjunction with polymeric osteopontin. Although high molecular weight polymeric osteopontin induced cell adhesion independently of TG2, these effects were only comparable to monomeric osteopontin and significantly less than polymeric osteopontin and TG2 complexes. The potent effects induced by polymeric osteopontin and TG2 may be attributed to the combined effects of high molecular weight polymers and TG2 reinforcement of cell surface receptor interactions. Cell surface TG2 has been shown to function as an integrin-binding adhesion co-receptor for FN through direct non-covalent interactions with β1 and β3 integrin subunits. The formation of stable ternary complexes with integrins and FN is stabilized by TG2 where it functions as a bridge between integrins and FN and thereby enhances these cell-matrix interactions[119]. Janiak et al. demonstrated that cell surface TG2 induces integrin clustering independently of integrin-ligand interactions[168]. Thus, the observed effects of enhanced biological activity in the presence of polymeric osteopontin and TG2 may be attributed to the combined effects of the composites, and reinforcement of cell surface receptor interactions by TG2 mediated integrin clustering.

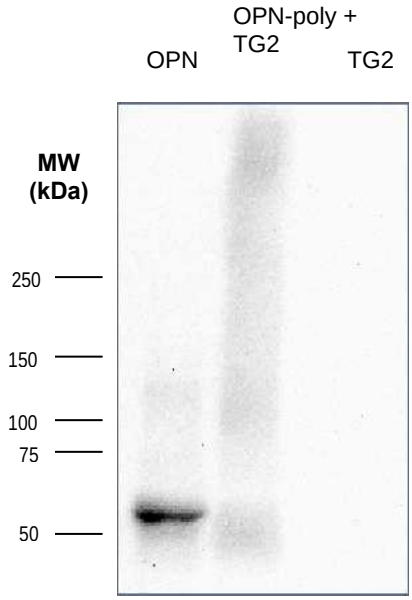

Figure 3.1: Multimerization of recombinant osteopontin (OPN) by transglutaminase 2 (TG2). Following osteopontin polymerization, 0.5µg of the resultant proteins were loaded into each lane of a 8-12% Tris-Glycine gel. Proteins were transferred onto a polyvinylidene fluoride membrane following a one and half hour transfer at 100V on ice. Unoccupied protein binding sites on the membrane were blocked by placing the membrane in a blocking buffer (3% bovine serum albumin in Tris-tween buffered saline) and then probed with an anti-osteopontin monoclonal antibody (1µg/mL) followed by an HRP conjugated secondary antibody (1:2000). [OPN-poly = Polymeric osteopontin]

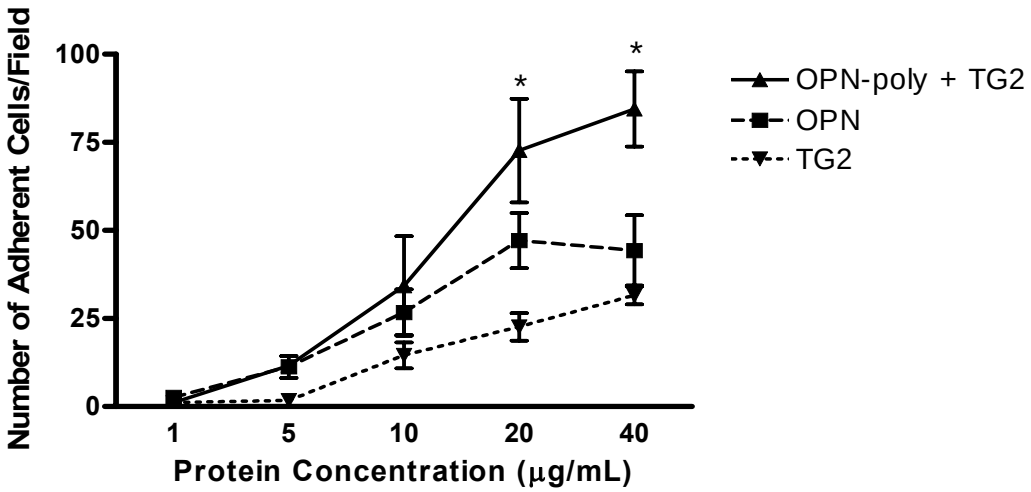

Figure 3.2: Adhesion of human MSCs to monomeric and polymeric osteopontin as well as TG2 adsorbed to plastic. 5000 cells in 100μL of serum-free media were plated onto wells coated with varying concentrations of monomeric osteopontin (■), polymeric osteopontin and TG2 (▲) or TG2 alone (▼). Non-adherent cells were removed and adherent cells in five fields were quantified. Each plot represents the mean (±SEM). [n=6; *p<0.05; polymeric vs. monomeric osteopontin]

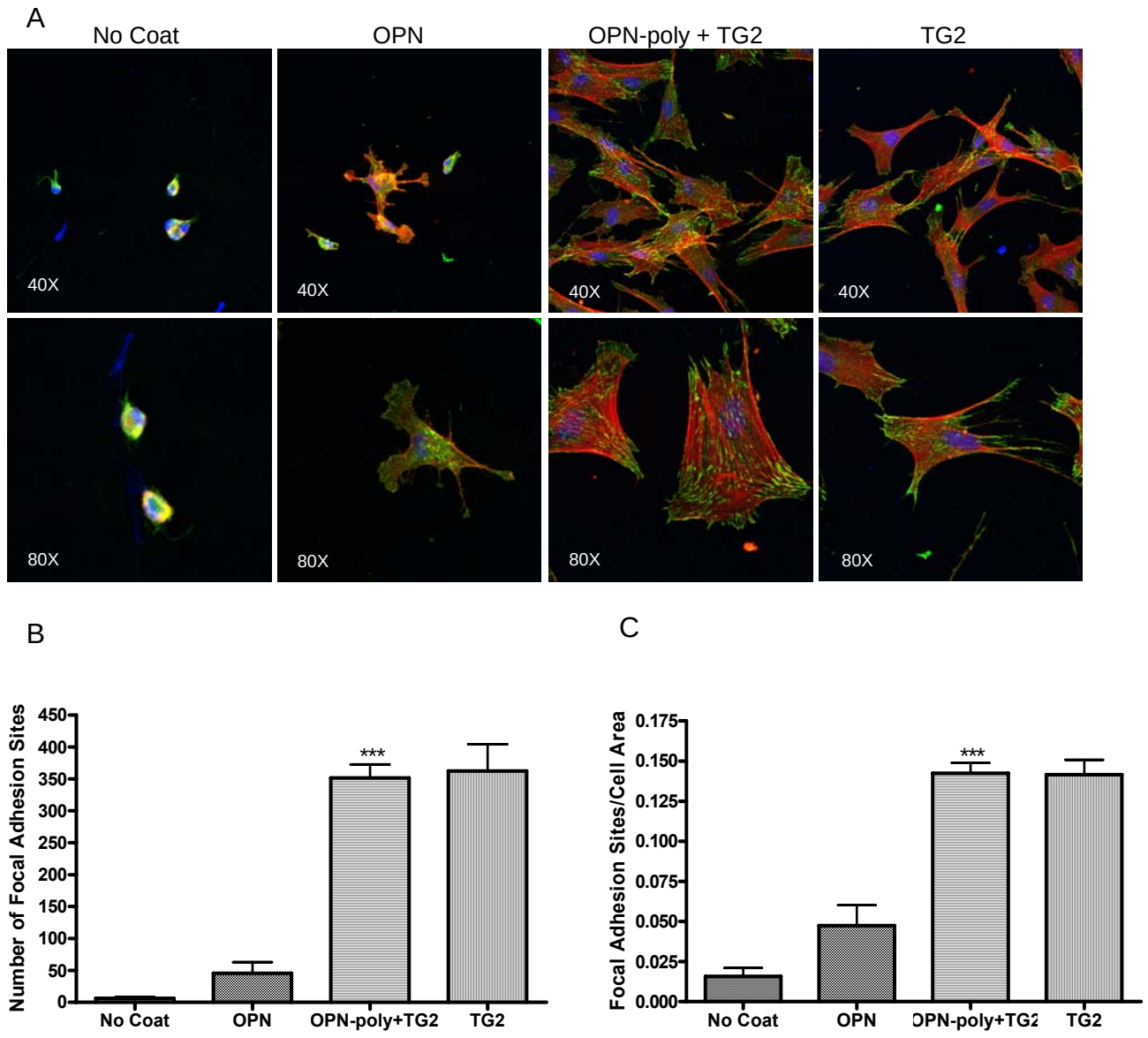

Figure 3.3: Cytoskeletal network morphology and focal contact formation of human MSCs in the presence of no adhesion molecules (no coat), monomeric osteopontin (OPN), polymeric osteopontin and TG2 (OPN-poly + TG2) or TG2 alone. 8-well chamber well glass slides were coated with either 0.1% BSA in PBS (no coat), 20µg/mL monomeric osteopontin, 20µg/mL polymeric osteopontin and 40µg/mL TG2 or 40µg/mL TG2 alone. $1.1x10^4$ cells in serum-free media were plated in each well and allowed to adhere for 2 hours. Non-adherent cells were then removed and adherent cells were stained for actin filaments using Alexa 546 conjugated phalloidin, nuclei with ToPro3, and vincullin with an anti-vincullin primary antibody followed by a biotinylated anti-mouse IgG secondary antibody and subsequently streptavidin conjugated Alexa Fluor 488. Samples were assessed with confocal microscopy (A). Focal contact sites were then quantified using image analysis software. Images captured by confocal microscopy were examined for number of focal adhesion sites (B) and number of focal adhesion sites per cell area (C) in 20 cells. Each plot represents the mean (±SEM). [n=3; ***p<0.001, polymeric vs. monomeric osteopontin]

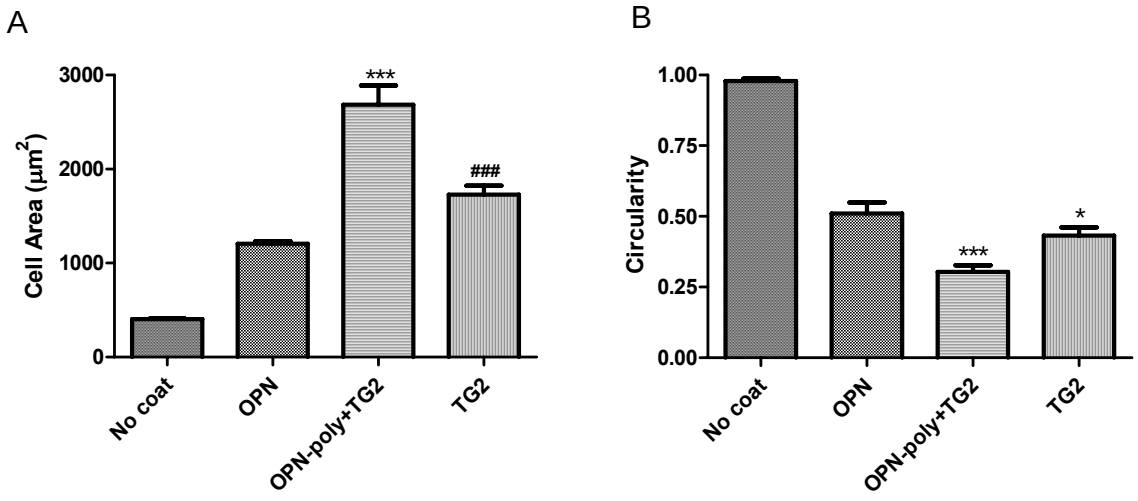

Figure 3.4: Morphometry of human MSCs in the presence of no adhesion molecules (No coat), monomeric osteopontin (OPN), polymeric osteopontin and TG2 (OPN-poly+TG2) or TG2 alone adsorbed to plastic. 5000 cells in 100μL of serum-free media were plated onto wells coated with either 0.1% BSA in PBS (no coat), 20μg/mL monomeric osteopontin, 20μg/mL polymeric osteopontin and 40μg/mL TG2 or 40μg/mL TG2 alone. Cells in five different fields (per well) were examined by brightfield microscopy and subsequently ImageJ software where each individual cell was traced and examined based on cell area (A) and circularity (B). A minimum of 50 cells per field were traced. Each plot represents the mean (±SEM) (n=6; A: ***p<0.001, monomeric vs. polymeric OPN, ###p<0.001, polymeric OPN+TG2 vs. TG2; B: **p<0.01, monomeric vs. polymeric OPN).

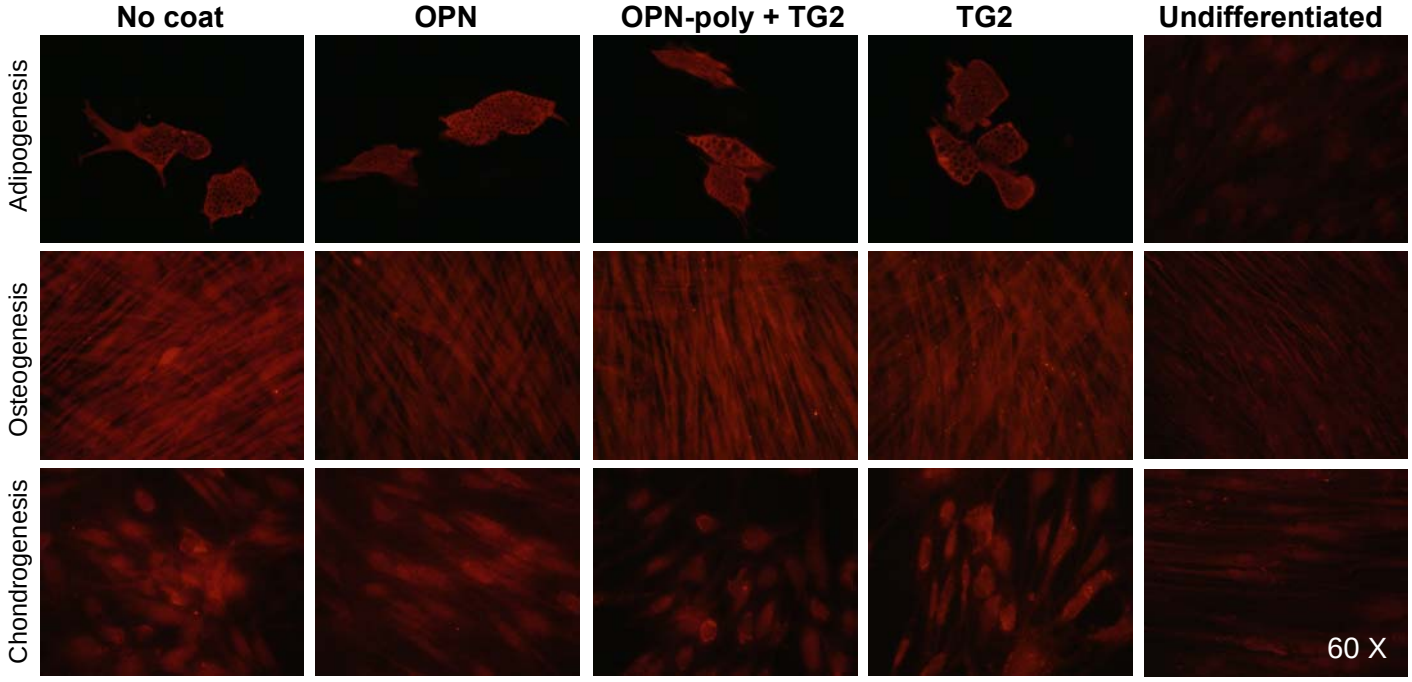

Figure 3.5: Pluripotency of human MSCs in the presence of no adhesion molecules (no coat), monomeric osteopontin (OPN), polymeric osteopontin and TG2 (OPN-poly + TG2) or TG2 alone. 8-well chamber well glass slides were coated with 0.1% BSA in PBS (no coat), 20µg/mL monomeric osteopontin, 20µg/mL polymeric osteopontin and 40µg/mL TG2 or 40µg/mL TG2 alone. $1.1x10^4$ cells/cm^2 were plated in each well and cultured in standard cell culture media (undifferentiated) or adipogenic, osteogenic or chondrogenic supplemented media for 21 days. Differentiated and undifferentiated cells were probed with polyclonal anti-Fatty Acid Binding Protein-4 antibody (adipocytes), monoclonal anti-osteocalcin antibody (osteocytes) or polyclonal anti-aggrecan antibody (chondrocytes) followed by a Rhodamine Red-conjugated donkey anti-goat or donkey anti-mouse secondary antibody. Samples were assessed with fluorescent microscopy.

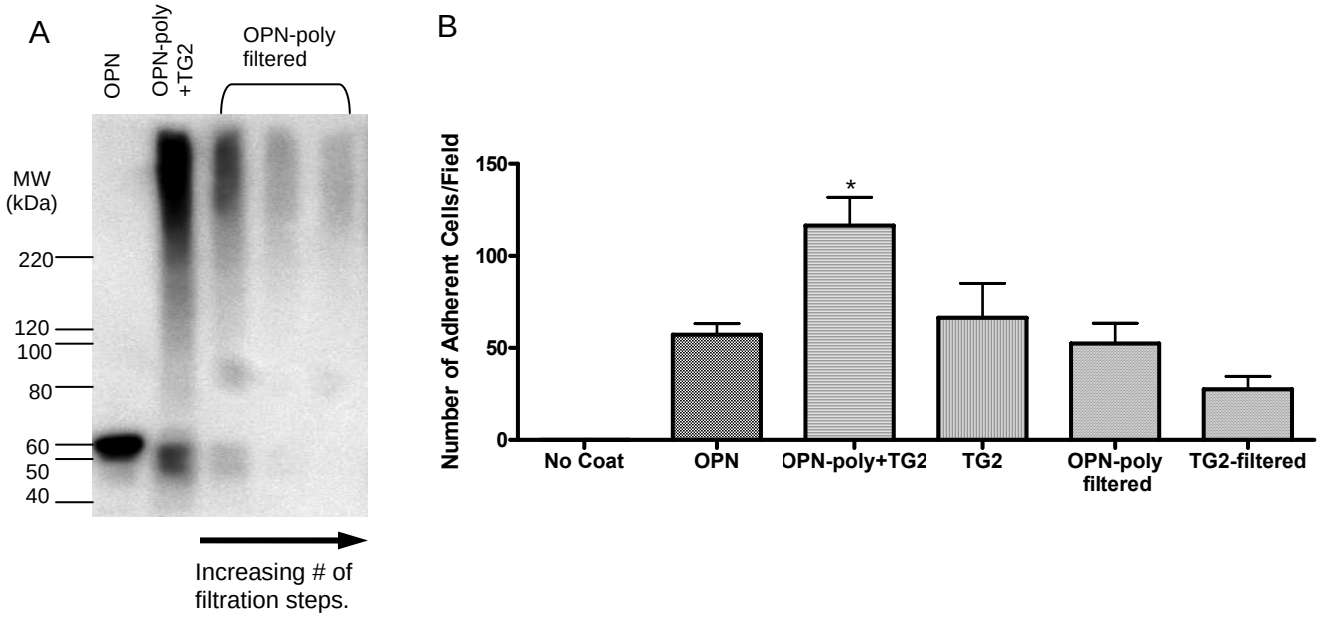

Figure 3.6: Confirming removal of low molecular weight proteins from polymeric osteopontin preparation and investigating adhesion of human MSCs to polymeric osteopontin independent of residual TG2 and osteopontin monomers. (A) Following polymerization of osteopontin by TG2, samples were filtered with the Microcon Centrifugal Filtration device with a molecular weight cut-off of 100kDa. Low molecular weight proteins would pass through the membrane by spinning samples at 3000g for 3 minutes at room temperature. High molecular weight proteins were recovered from the device by inverting the membrane into a new eppendorf tube and spinning at 1000g for 3 minutes at room temperature. These filtration and recovery steps were conducted a total of three times. Removal of low molecular weight proteins was confirmed by western blotting and probing with a monoclonal anti-osteopontin antibody. (B) Adhesion of human MSCs in the presence of no adhesion molecules (No coat), monomeric osteopontin (OPN), polymeric osteopontin and TG2 (OPN-poly+TG2; unfiltered or filtered high molecular weight samples) or TG2 alone (unfiltered or filtered samples) adsorbed to plastic. 5000 cells in 100µL of serum-free media were plated onto wells coated with either 0.1% BSA in PBS (no coat), 20µg/mL monomeric osteopontin, 20µg/mL polymeric osteopontin and 40µg/mL TG2 or 40µg/mL TG2 alone. Each plot represents the mean (±SEM). (*$p<0.05$; OPN vs. OPN-poly+TG2 and OPN-poly filtered vs. OPN-poly+TG2; n=3; each experiment is done in duplicate samples).

4.0 Project II: Matrix Supplemented Encapsulation to Reduce Multipotent Stromal Cell Death

4.1 Study Aims and Rationale

MSC microencapsulation can be employed as a novel cell delivery strategy to administer a suspension of individual encapsulated stem cells to the target tissue by intravascular injection. It is believed that microcapsules supplemented with matricellular proteins maintain the therapeutic potential of stem cells by engaging cell surface receptors, thus avoiding anoikis, as well protecting the cells from insults associated with injection and delivery to the target tissue. Encapsulated stem cells are filtered by and lodge into the microvasculature. These capsules are designed to provide temporary shelter (i.e cocoons), from which the cells can emerge by migration within hours to penetrate and engraft the surrounding tissue. The cocoons can also be functionalized by addition of other proteins/factors in order to maintain a desired state of differentiation of MSCs, and to promote the regeneration of functional tissue. Microcapsules, prepared with agarose gel supplemented with matricellular proteins (FN and FG), have previously been shown in previous studies to enhance survival and pulmonary retention of syngeneic rat MSCs[146].

To optimize our MSC encapsulation procedures, an adhesive glycoprotein (osteopontin) and an extracellular matrix cross-linking enzyme (TG2) were incorporated into the microcapsules. Additional matrix supplementation was considered as a means to enhance cell survival by engaging multiple cell surface receptors, while stabilizing the provisional matrix. After confirming the direct biological effects of polymeric osteopontin on MSCs, the following appropriate hypotheses were formulated.

Specific Hypotheses:

1) Supplementation of microcapsules containing FN and FG with polymeric osteopontin and TG2 will prevent MSC anoikis.
2) Polymeric osteopontin and TG2 microcapsular supplementation will function synergistically with FN and FG to protect MSCs from anoikis.

Project Aims:

To investigate the effects of incorporation of osteopontin into microcapsules with and without FN and FG on MSC survival in suspension cultures.

4.2 Methods

Human MSCs, passage 3-5, were encapsulated in agarose gel microcapsule preparations containing various combinations of matrix and matricellular proteins. Encapsulated cells were maintained in suspension cultures generated with PolyHEMA coated substrates. Apoptotic cells were quantified using flow cytometry assessment of Annexin V and Propidium Iodide stained samples, where $1.0x10^4$ events were examined. Apoptosis trends were confirmed using a fluorimetric caspase assay. All apoptosis assays were conducted three times. Molecular organization of matrix proteins encompassed in microcapsule preparations were investigated with fluorescently tagged proteins and fluorescent confocal microscopy. Detailed descriptions of corresponding methods are provided in Section 2.0.

4.3 Results

4.3.1 Reduced apoptosis of encapsulated MSCs

Cells were encapsulated and maintained in a suspension culture for 24 hours before quantifying the percentage of apoptotic cells with Annexin V/Propidium iodide staining and flow cytometry. Apoptosis studies were confirmed with fluorimetric caspase activity assays of encapsulated MSCs maintained in suspension cultures for 3 hours. MSCs in the newly supplemented microcapsules with either monomeric osteopontin, polymeric osteopontin with TG2, or TG2 alone were studied.

As shown in Figure 4.1A, microencapsulation of cells with FN and FG together with polymeric osteopontin and TG2 resulted in a dramatic reduction in apoptosis of nearly 50% compared to FN/FG alone (14.0±2.34% vs. 28.2±3.22%, respectively; $p<0.05$; $n=3$). FN/FG microcapsules further supplemented with either monomeric osteopontin or TG2 reduced apoptosis by 8% and 33%, respectively compared to FN/FG microcapsules, however, these differences were not statistically significant. Reduction in apoptosis of MSCs in FN/FG microcapsules supplemented with polymeric osteopontin and TG2 was confirmed with a fluorimetric caspase activity assay which revealed a 40% reduction in caspase activity in MSCs compared to cells in FN/FG microcapsules (Figure 4.1B).

To investigate the underlying mechanism resulting in this reduction in apoptosis, MSCs were encapsulated without FN and FG. Microcapsules were prepared as described above in one of four possible combinations of matrix proteins: no supplementation, monomeric osteopontin (20μg/mL), polymeric osteopontin and TG2 (20μg/mL and 40μg/mL respectively) or TG2 (40μg/mL). Encapsulated cells were maintained in the poly-Hema suspension cultures at 37°C and 5% CO_2 for 24 hours before being collected and spun at room temperature for 5 minutes at 450g. Cell survival was examined with Annexin V and Propidium Iodide staining and flow cytometry (as described). Interestingly, in the absence of FN/FG, none of the matrix supplementations appeared to have an effect on the percentage of apoptotic cells relative to the non-supplemented agarose capsules (Figure 4.1C).

4.3.2 Change in Structure of Microcapsule Matrix by Transglutaminase 2

To confirm that the different morphological forms of osteopontin, monomeric and polymeric, are present in the microcapsules and to examine their distribution within the

microcapsules, osteopontin was fluorescently labeled and assessed with confocal microscopy. Staining of osteopontin following the preparation of microcapsules confirmed that both monomeric and polymeric osteopontin are retained in microcapsules regardless of whether these also contain FN/FG (Figure 4.2). Microcapsules containing monomeric osteopontin with or without FN/FG exhibited relatively even distribution of a lightly speckled pattern of osteopontin in the hydrogel matrix. In the absence of FN/FG, polymeric osteopontin showed a distribution that was identical to that of its monomeric form. On the other hand, in the presence FN/FG, polymeric osteopontin formed large clusters or aggregates of osteopontin that were unevenly distributed throughout the microcapsule. It is of interest to note that this was the only preparation that resulted in a significant reduction in apoptosis of encapsulated cells (Figure 4.1A).

To further investigate the distribution of matrix proteins in the microcapsules, fluorescently tagged fibrinogen was incorporated in the microcapsule preparations. Assessment of samples with confocal microscopy revealed large clusters of FG in FN/FG microcapsules supplemented with either polymeric osteopontin and TG2 or TG2 alone (Figure 4.3). This pattern of distribution contrasted distinctly from the distribution pattern of FG in the microcapsules supplemented only with FN/FG with or without with monomeric osteopontin. Clustering of FG in microcapsules containing TG2 paralleled the distribution pattern of polymeric osteopontin in microcapsules containing FN/FG (Figure 4.2), suggesting that TG2 may have crosslinked some of the FG content in the FN/FG microcapsules. Such crosslinking activity may have also contributed to the aggregation of polymeric osteopontin noted in the FN/FG microcapsules (Figure 4.2).

4.4 Discussion

Additional matrix supplementation of agarose containing FN/FG with polymeric osteopontin and TG2 resulted in a microcapsule that induced a significant (~50%) reduction in the percentage of cells undergoing apoptosis in suspension culture relative to FN/FG microcapsules. This dramatic difference was unique to this particular combination of matrix proteins, and was not seen in the presence of any of the individual components, thereby suggesting a potential synergistic activity between these matrix proteins.

Agarose microcapsule supplementation with the matrix proteins, FN/FG, has previously been shown to enhance MSC viability in suspension cultures relative to non-supplemented microcapsules[146]. Improved viability was believed to be a result of immobilization of the specific matrix proteins in microcapsules rather than general protein supplementation[146]. Although further supplementation of FN/FG microcapsules with polymeric osteopontin and TG2 resulted in a minor 6% increase in total protein content relative to FN/FG microcapsules, increasing protein content to a similar degree by the addition of monomeric osteopontin or TG2 did not result in a significant reduction in apoptosis of encapsulated cells grown in suspension culture relative to FN/FG microcapsules. Thus, this suggests that reduction in apoptosis was due to the combination of the specific molecular components of the engineered microenvironment and is not likely due to increased protein supplementation.

Molecular organization of matrix proteins in microcapsules may have played an important role in the overall effect. Confocal microscopy assessment of fluorescently stained osteopontin in microcapsules revealed a distinct pattern of distribution of polymeric OPN, which formed large clusters within microcapsules supplemented with FN/FG. This was in stark contrast to the lightly speckled pattern found in the microcapsules of polymeric OPN in the absence of FN/FG or mononeric OPN. The macroaggregation pattern of polymeric OPN was uniquely associated with a reduction in apoptosis of encapsulated MSCs, compared to all other combinations of matrix proteins, which exerted negligible effects on cell survival in suspension culture. Large osteopontin clusters were present solely in microcapsules inducing a significant reduction in MSC apoptosis, suggesting that matrix distribution is an important factor regulating apoptosis. This molecular arrangement may have functioned to concentrate integrin binding sites and thereby promoted greater integrin clustering which is known to prevent apoptosis[82].

Examination of matrix protein distribution in microcapsules using fluorescently tagged FG displayed a clustering of FG in microcapsules containing TG2. This distribution distinctly contrasted the distribution pattern of FG in microcapsules lacking TG2 content (ie. FN/FG microcapsules and FN/FG microcapsules further supplemented with osteopontin), suggesting TG2 activity may have polymerized some of the FG content in the FN/FG microcapsules. This polymerization may have also promoted the aggregation of polymeric osteopontin noted in the FN/FG microcapsules and thereby stabilizing the hydrogel matrix to promote greater cell adhesion. Transglutaminases such as Factor XIIIa have been shown to catalyze the formation of FG oligomers[169-172] as well as FN-FG hybrid multimers[167,173]. Crosslinked FN and FG dimers can crosslink with other hybrid dimers via their FG γ-chains and generate larger molecular weight oligomers[167]. Similar to polymeric osteopontin, these crosslinked proteins may have enhanced biological activity relative to their monomeric composites. Conformational changes of crosslinked proteins have been suggested to concentrate cell surface receptor ligand-binding sites and thereby enhance interactions with integrins by promoting integrin clustering, or may even expose cryptic epitopes recognized by various cell surface receptors[101]. In addition, these oligomers may have induced the aggregation of polymeric osteopontin that in turn concentrated numerous integrin binding sites, leading to the formation of integrin clusters which prevent apoptosis. Thus, TG2 likely plays an important role in mediating the polymerization and subsequent clustering of matrix proteins in microcapsules. These effects may be serving to enhance cell-matrix interactions by inducing a concentration of integrin binding sites which in turn is promoting greater integrin clustering and thereby preventing MSC apoptosis[82].

These observations are also consistent with the notion that both the molecular composition and the physical state of matrix play an important role in cell-matrix adhesion and the subsequently activated signal transduction pathways. Previous studies have shown that the morphological presentation of matrix proteins is critical to the activation of cell survival signaling pathways[174,175]. Encapsulated MSCs treated with soluble FN and FG in suspension cultures displayed greater cell death than cells in the presence of these proteins as solid-state forms in microcapsules[146]. Katz et al. proposed that while integrins associate with their corresponding solid-state ligands to form adhesion complexes, physical properties of the surrounding microenvironment such as rigidity, regulate local tension at adhesion sites and

further promote the formation of focal adhesion contact sites[97]. Crosslinking of FN to a substrate, rather than it being adsorbed where it would be more pliable, exhibits an exaggeration of focal adhesions[97]. Changes in cellular response to the rigidity of the surrounding matrix may be a product of increased intracellular tension against the fixed substrate[96]. Experimental application of tension near focal adhesion sites demonstrates an enlargement of these complexes and thus reaffirms the dynamic relationship between the forces on adhesion and their size and function[176-178]. Therefore, clustering of matrix proteins in microcapsules may also be functioning as stabilized anchorage sites promoting exaggerated focal contact sites due to increased tension surrounding specific integrin-ligand binding. These exaggerated cell-adhesion sites may in turn amplify activation of appropriate signaling pathways to prevent adhesion dependent apoptosis, anoikis.

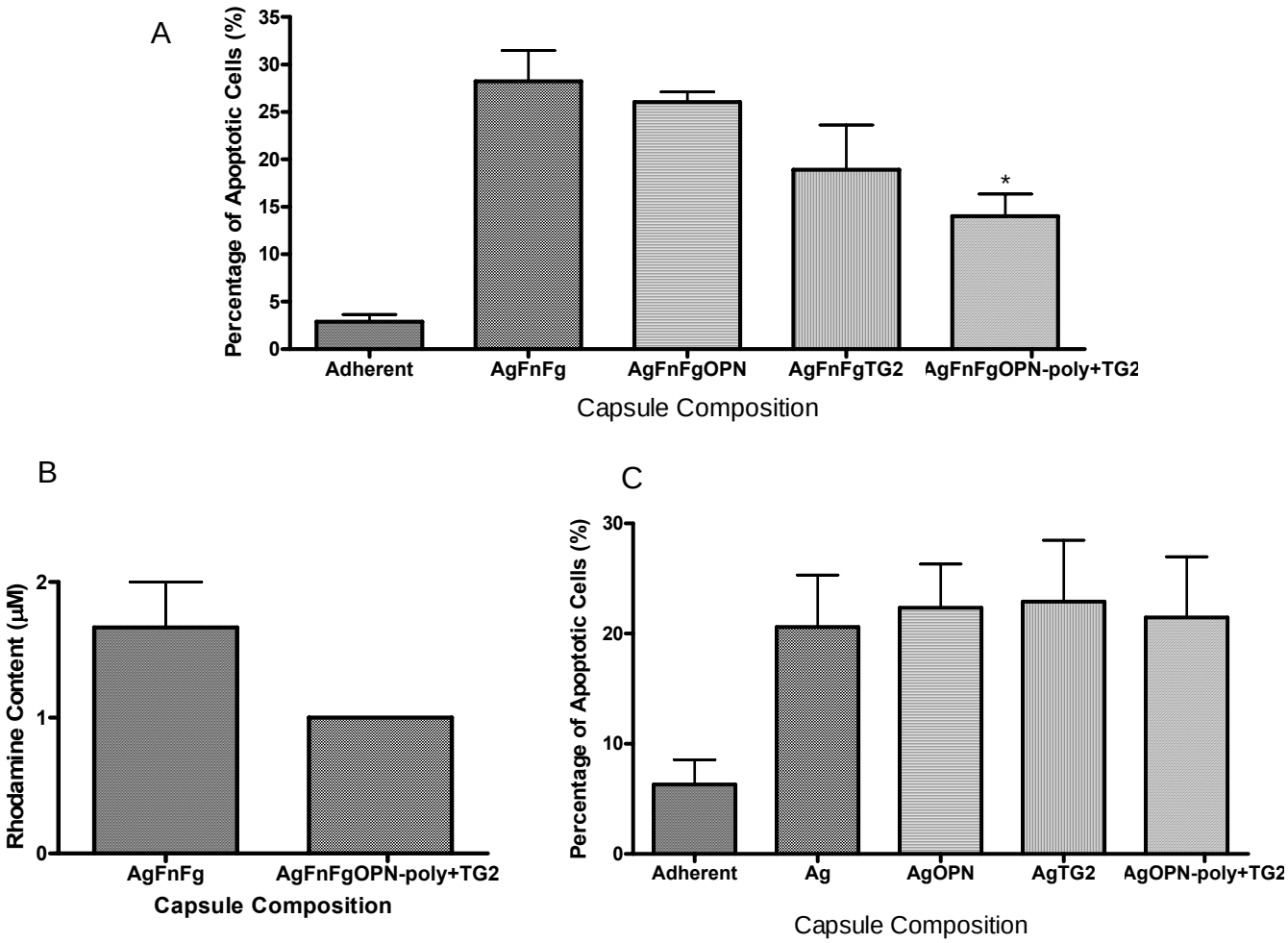

Figure 4.1: Apoptosis and caspase activity of human MSCs in an adherent or encapsulated culture. (A & B) 1.6x10^6 cells/1 mL of capsule preparation were in encapsulated in either the capsules supplemented with only fibronectin (80µg/mL) and fibrinogen (800µg/mL) or further supplemented with monomeric osteopontin (20µg/mL), polymeric osteopontin and TG2 (20µg/mL and 40µg/mL, respectively) or TG2 (40µg/mL). (C) 1.6x10^6 cells/1 mL of capsule preparation were encapsulated in microcapsules with either no matrix protein supplementation or supplemented with monomeric osteopontin (20µg/mL), polymeric osteopontin and TG2 (20µg/mL and 40µg/mL, respectively) or TG2 (40µg/mL). (A&C) Encapsulated cells were maintained in suspension culture for 24 hours and percentage of apoptotic cells were quantified by Annexin V and Propidium Iodide staining and flow cytometry. (B) Encapsulated cells were maintained in suspension culture for 3 hours and the relative level of apoptosis was quantified with a fluorimetric caspase activity assay. Fluorimetric measurement of free fluorochrome (rhodamine) content is proportional to the concentration of activated caspases. Each plot represents the mean (±SEM; n=3). [Ag = Agarose, Fn = Fibronectin, Fg = Fibrinogen, OPN = Monomeric osteopontin, TG2 = Transglutaminase 2, OPN-poly = Polymeric osteopontin] (A: n=3; *p<0.05, AgFnFg vs. AgFnFgOPN-poly+TG2).

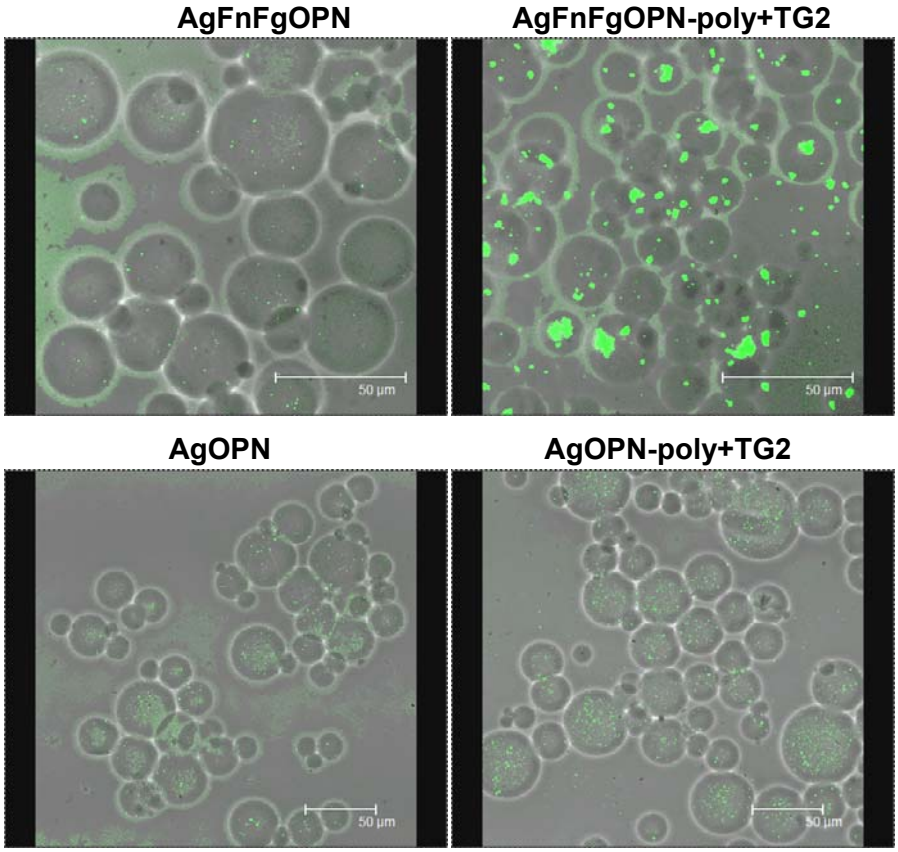

Figure 4.2: Fluorescently labeled monomeric and polymeric osteopontin in microcapsules. Monomeric osteopontin (20µg/mL; OPN) or polymeric osteopontin and TG2 (20µg/mL and 40µg/mL, respectively; OPN-poly+TG2) were incorporated in standard microcapsules (top panel) containing fibronectin (80µg/mL; Fn) and fibrinogen (800µg/mL; Fg) or microcapsules without fibronectin and fibrinogen (bottom panel). Osteopontin immobilized the microcapsules was probed with a monoclonal anti-osteopontin primary antibody followed by a biotinylated anti-mouse IgG secondary antibody and subsequently streptavidin conjugated Alexa Fluor 488. Samples were assessed with confocal microscopy.

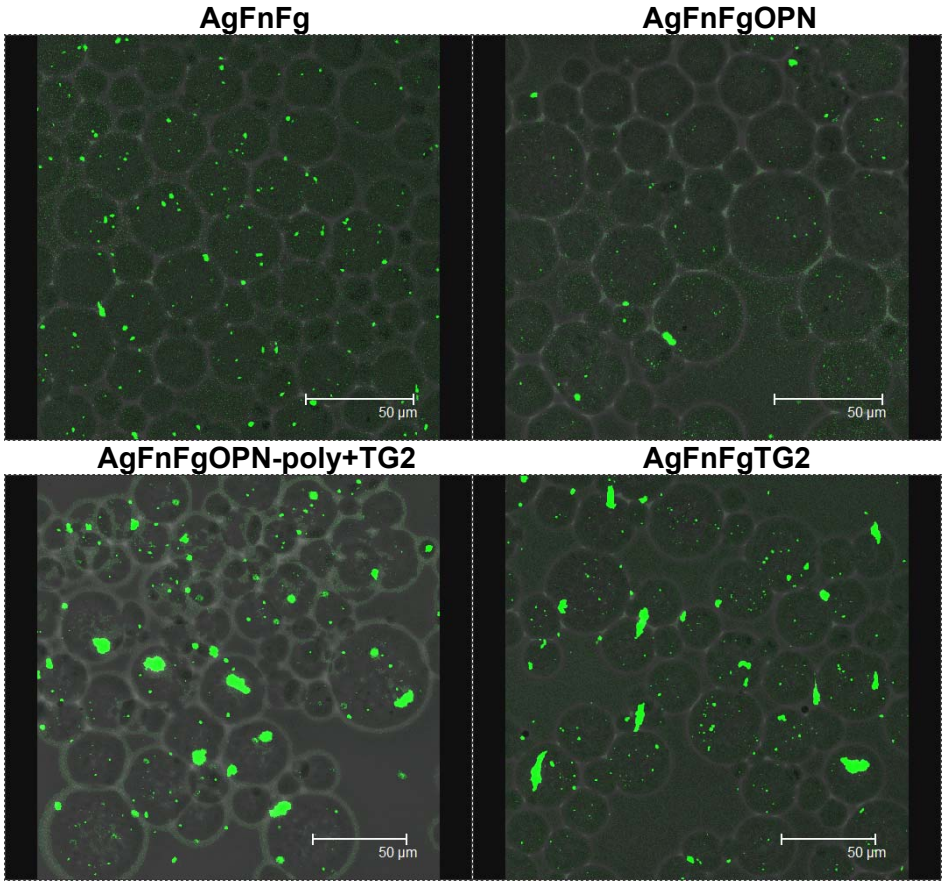

Figure 4.3: Oregon-green conjugated fibrinogen in microcapsules. Standard microcapsules containing fibronectin (80µg/mL) and fibrinogen (800µg/mL) and further supplemented with either monomeric osteopontin (20µg/mL) or polymeric osteopontin and TG2 (20µg/mL and 40µg/mL, respectively) were prepared with Oregon-green conjugated fibrinogen. Samples were assessed with confocal microscopy.

4.0 General Discussion

Microcapsules, prepared with agarose gel supplemented with matrix proteins (FN/FG), have been shown in previous studies to enhance survival and pulmonary retention of syngeneic rat MSCs[146]. In this thesis, I have demonstrated that osteopontin can engage MSCs and this interaction is dramatically heightened by TG2-mediated polymerization of osteopontin. I have also shown that MSCs are protected from anchorage-dependent apoptosis, anoikis, by the synergistic engagement of FN, FG, and polymeric osteopontin and TG2 in the agarose gel-based microcapsules. Maximizing cell viability in suspension cultures is an important prerequisite for eventually enhancing transplanted cell engraftment. Increasing the survival of exogenously administered cells in target tissues has been attempted using numerous approaches, and improved cell survival has been shown in a number of studies to result in enhanced therapeutic outcome[62,179-181]. Many of these strategies employ gene modification of exogeneous cells, often designed to overexpress anti-apoptosis factors (i.e Akt), which may not be readily translated to a clinical therapy in part due to safety concerns relating to possible induction of neoplasia[182]. In contrast, the MSC microcapsule system represents a novel technique that does not present any major safety concerns and may be applied across organ systems, with potential for greater efficacy.

TG2 mediated polymerization of osteopontin generated polymers of variable size, resulting in the production of a heterogeneous mixture of multimeric complexes. Interestingly, these polymeric complexes demonstrated more potent effects than that of monomeric osteopontin. As with other matricellular proteins, monomeric osteopontin induces an intermediate state of cell adhesion, characterized by cell elongation and a lack of well defined stress fibers and focal adhesion sites. This is consistent with the unique adhesive activity of matricellular proteins, such that they induce an intermediate state of cell adhesion[125]. Cell morphology correlating to this intermediate state is similar to that of cells strongly adhered to a surface; however the cytoskeletal structure remains fluid and does not display strong definition. Cells adhere rather loosely to substrates enriched with monomeric matricellular proteins, and thus do not form focal contact sites which would otherwise function to localize the cytoskeletal network, leading to stress fiber formation[125]. In contrast,

TG2 mediated polymerization of osteopontin alters the cellular responses to become similar to that of traditional matrix proteins. Thus, osteopontin polymers induce more potent cell adhesion, cell spreading along with the formation of numerous well defined focal adhesion sites. These adhesion sites function to localize actin filaments and induce well defined stress fiber formation. Therefore, it is likely that conformational changes in osteopontin structure induced by TG2 mediated polymerization[120], alter the matricellular protein such that 1) it has more potent capacity to mediate cell-matrix interactions than monomeric osteopontin; 2) it acquires the ability to serve a structural role similar to traditional matrix proteins.

In the context of the newly supplemented microcapsule system, where the provisional matrix is composed of FN, FG, polymeric osteopontin and TG2, cells are provided with large aggregates of matrix and matricellular proteins. This particular combination of adhesion molecule ligands resulted in a striking and significant (~50%) reduction in the percentage of encapsulated apoptotic cells relative to the FN/FG supplemented microcapsules. As evidenced by the respective changes in cell adhesion, morphology and cytoskeletal structure, polymeric osteopontin may take on the structural role of a traditional matrix protein, serving as a substrate for strong cell adhesion. In the microcapsule, polymeric osteopontin may function to induce and maintain elongated cell morphology coupled with prevalent focal contact sites and stress fiber formation. This extended morphology and stimulation of intracellular signalling pathways may be contributing to the enhanced resistance to apoptosis which was achieved by encapsulating MSCs in agarose containing these protein components. It is known that prevention of anoikis is not achieved by integrin signalling alone, and that cell elongation is also an essential component in maintaining survival[183]. In addition, the presence of TG2 in microcapsules was also a likely contributor to reducing MSC apoptosis. TG2 exhibits adhesive qualities through its non-covalent interactions with with β1 and β3 integrins in focal adhesion sites. These interactions have been shown to allow TG2 to serve as a bridge between respective integrins and ligands including FN, and thereby enhancing cellular interactions with matrix proteins[119]. Thus, the presence of TG2 in microcapsules containing FN, FG and polymeric osteopontin may have reinforced integrin binding with these respective matrix proteins, and in turn stimulating prosurvival signalling pathways.

As mentioned, enhanced resistance of MSCs to apoptosis was unique to a particular combination of matrix proteins (FN, FG, polymeric osteopontin and TG2) and suggests potential synergistic activity of the matrix proteins. Molecular organization of these matrix proteins in microcapsules may have contributed to this synergistic activity. Aggregation of polymeric osteopontin and FG exhibited in microcapsules in the presence of FN/FG and TG2 may have served to enhance cell-matrix interactions by concentrating integrin binding sites. This may have in turn promoted greater integrin clustering and thereby enhancing MSC resistance to anoikis[82]. Molecular organization of matrix proteins is likely mediated by TG2 activity, and this may have also altered physical properties of the surrounding microenvironment, such as matrix rigidity. Increased substrate rigidity regulates local tension at adhesion sites and further promotes the formation of focal adhesion contact sites[97] Changes in cellular response to the rigidity of the surrounding matrix may be a product of increased intracellular tension against the fixed substrate[96]. The dynamic relationship between the adhesion forces and the size and function of focal adhesion sites has been confirmed with studies employing experimental application of tension near focal adhesion sites. These studies have demonstrated an enlargement of these complexes with increased vinculin expression[176-178]. Therefore, polymerization and subsequent clustering of matrix proteins in microcapsules may also act to stabilize anchorage sites and enhance focal contact sites due to increased tension surrounding specific integrin-ligand binding. These exaggerated cell-adhesion sites may in turn amplify activation of appropriate signaling pathways and thereby enhancing MSC resistance to anoikis.

Integrin-mediated response to tension in the microcapsule system can be extended further than just the rigidity of the matrix encompassing cells. The mechanosensory nature of integrins may also be responding to mechanical forces applied during the process of microcapsule preparation, thereby leading to strengthened integrin mediated adhesion[184]. Integrin mediated binding is mechanosensitive responding to an applied force by reinforcing these binding sites to counteract the force[185]. Within minutes, adhesion sites enlarge by the recruitment of additional integrins, and thereby extending in the direction of the applied force[177,186]. In the context of the microcapsule system, force is applied repeatedly in the preparation of microcapsules. Multiple spinning cycles are used to isolate microcapsules

from the inert silicone oil used to initially form microcapsules through differences in surface tensions between the aqueous hydrogel and oil solutions. During the spinning cycles, cell interactions with matrix proteins may undergo transformations from relaxed binding to stronger interactions defined as tension binding in response to the tension experienced[184]. These strengthened interactions may stimulate a variety of downstream intracellular signaling, such as the activation of focal adhesion kinase (FAK) which has been shown to require actin and myosin-dependent tension[186,187]. FAK activation plays an important role in the suppression of anoikis[188], and thus may further contribute to MSC resistance to anchorage-dependent apoptosis. This approach to improving MSC resistance to apoptosis using matrix supplemented microcapsules is novel, however, the objective is shared by others also seeking to minimize death of transplanted cells.

Various alternative approaches have been explored for prevention of apoptosis following cell transplantation. Many of these strategies encompass genetic modification of cells, in particular the overexpression of antiapoptotic factors. Li et al. (2007) demonstrated in a recent study that transfection of MSCs with an antiapoptotic *Bcl-2* gene protected many of these cells from apoptosis in vitro and thereby promoted significantly enhanced long-term cell engraftment in post-infarcted myocardium[179]. These results were also coupled with improved functional myocardial recovery along with greater capillary density and reduced infarct size[179]. Akt, another important antiapoptotic gene, has been employed in a similar manner as Bcl-2 to genetically modify MSCs to exhibit greater apoptotic resistance[180]. MSCs transduced to overexpress Akt demonstrate enhanced capacity to overcome apoptosis and exert their therapeutic effects by up-regulating the expression of VEGF, fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) under both normoxic and hypoxic conditions[180]. Secretion of these factors by MSCs in the infracted myocardium, resulted in pro-angiogenic, cardioprotective and inotropic effects, contributing to the notion that transplanted MSCs exert their therapeutic actions through paracrine activity[180]. Interestingly, both these studies highlight the dramatic therapeutic potential of MSCs engineered with enhanced antiapoptotic capacity correlating to enhanced post-transplantation survival. Greater resistance to apoptosis and in turn, increased cell engraftment, translated to greater therapeutic outcome in each respective study.

Additional means of enhancing survival of transplanted cells have focused on promoting cell adhesion and thereby preventing anoikis, using strategies similar to the microcapsule system. Song et al. recently reported significant improvement in retention of MSCs that were genetically engineered to overexpress TG2, in infarcted myocardium, attributed to an increased formation of focal adhesion complexes[181]. TG2 transfected MSCs displayed 33.1% enhanced adhesiveness in a cardiac fibroblast-derived three dimensional matrix, cardiogel, relative to control MSCs[181]. In addition, TG2-MSC therapy of infarcted myocardium normalized systolic and diastolic cardiac function, and this effect was more pronounced in transfected cells compared to MSCs alone[181]. Laflamme et al. also demonstrated the role of anoikis in transplanted cell death, however their study did not employ genetic modification of cells[62]. Human embryonic stem cell derived cardiomyocytes were shown to require a prosurvival supplemented matrix to prevent cell loss, and enhance cell engraftment and functional improvement of the infarcted myocardium[62]. Although the authors noted that cell survival was multi-factorial, the inhibition of anoikis was a prominent factor[62].

All together, these studies collectively emphasize that the therapeutic efficacy of MSCs in relation to infarcted myocardium is dependent on the in situ survival of exogenous cells in the damaged tissue[62,179-181,189,190]. Sites of myocardial injury present a hostile environment with hypoxia, inflammation and scarring challenging the capacity of cells to incorporate into the surrounding environment[179]. In all of the above mentioned studies, cells were transplanted directly into the cardiac tissue. Improvement of cardiac function is attributed to survival of transplanted cells and their subsequent migration to the peripheral infarct region where their therapeutic effects are noted[179-181]. However, administering exogenous cells by direct intramyocardial injection is a highly invasive means that may not be suitable for a wide range of patients. Hou et al. compared the efficacy of various cell delivery methods, including intramyocardial, intracoronary and interstitial retrograde coronary venous injection, demonstrated maximum myocardial cell retention by intramyocardial administration[191]. Although this method of cell delivery is feasible and widely used in numerous animal models, intramyocardial injection may not be a clinically relevant approach. Intracoronary injection on the other hand is limited by low levels of target-tissue cell engraftment[192,193].

The lack of cells detected in the infarcted myocardium is attributed to trapping of cells in small capillaries and loss of cells in the systemic circulation[192,193]. Intravenous delivery of cells represents a clinically relevant and minimally invasive technique, however it relies on cell homing to target an area of injury and therefore may result in lower levels of target-tissue cell engraftment[194]. Freyman et al.'s study comparing MSC injection by intravenous, intracoronary and endocardial delivery fifteen minutes post-MI demonstrated greatest 14-day cell retention by intracoronary infusion[195]. 14-days after post-MI cell delivery, 6% and 3% of intracoronary and endocardial (respectively) administered cells remained, while no exogenous cells were detected in infarcts receiving intravenous infusion[195].

The microencapsulation system represents a cell delivery strategy that can effectively deliver a suspension of individual encapsulated cells to the target tissue by intravascular injection. Microcapsules can lodge into the surrounding microvasculature, enclosed cells can subsequently migrate from the capsule and engraft in the surrounding tissue, while sustaining the therapeutic potential of the exogenous cells with matrix protein supplementation. In a manner similar to the effect of prosurvival matrices[62], microcapsules present immobilized adhesion proteins providing suitable anchorage sites for the enclosed cells and thereby trigger prosurvival signaling pathways[146]. However, unlike the previous report (Laflamme et al.), microcapsules can be delivered by intravascular injection and thus, can be easily administered to many different organ systems. In addition, the microcapsule system presents a provisional matrix surrounding each individual cell and thereby potentially maximizes the volume of matrix proteins per cell area. Agarose gel based microcapsules supplemented with 80μg/mL of FN and 800μg/mL FG, have demonstrated enhanced survival of MSCs attributed to the αvβ3 integrin mediated activation of the MAPK/ERK survival pathway[146].

Matrix protein supplemented microcapsules also represent a system that can potentially be utilized in conjunction with gene modification of cells for the over expression of antiapoptotic or survival factors. By encompassing genetically modified cells in matrix supplemented microcapsules, these therapeutic factors can potentially be targeted to specific tissues without the need for highly invasive delivery procedures. This may further enhance the therapeutic efficacy of cell-therapy and thus, may be of great interest for future studies.

However, it is of paramount importance to first investigate the therapeutic potential of the newly supplemented microcapsule system independently. Future studies must focus on examining the survival of MSCs post-transplantation and examining the therapeutic potential of these cells in conjunction with the constituent proteins encompassed in the newly supplemented microcapsule system. In addition to enhancing MSC resistance to apoptosis, these matrix and matricellular proteins may also contribute remedial qualities. With roles in wound healing, matrix remodeling as well as the interesting role of TG2 in vascular remodeling[107], this group of proteins may function concomitantly in the vasculature to maximize functional recovery of damaged tissue.

Future work may also be focused on investigating the mechanobiology of MSCs in the microcapsule system. As described, mechanical force and matrix physical properties can transform integrin binding affinity such that the strength of adhesion is exaggerated in response to applied force and increasing substrate rigidity. By employing microcapsules characterized by varying levels of matrix rigidity, insight can be gained regarding the role of provisional matrix rigidity in preventing anoikis. To investigate transformation of integrin binding affinity in response to mechanical stimulation, adhesive bonds must be examined prior to and after microcapsule spin cycles according to integrin crosslinking[184]. Shi and Boettiger demonstrated that the proportion of total α5β1 integrins displaying crosslinking activity is proportional to the number of adhesive bonds[196]. It should not be overlooked that in addition to understanding the cellular responses to the mechanical properties of the microcapsule system, it is also important to appreciate the underlying intracellular signaling pathways and cell surface receptors that augment MSC resistance to anoikis.

5.0 Conclusion

The microcapsule system encompasses a complex array of qualitites that concomitantly orchestrate the engagement of MSC interaction and subsequent buffering for the prevention of cell death. TG2 mediated polymerization of osteopontin appears to heighten the biological activity of this matricellular protein such that it dramatically enhances human MSC adhesion, elongation, and focal contact formation. In the microcapsule setting, enhanced resistance of human MSCs to anoikis is attributed to the synergistic activity of FN, FG, polymeric osteopontin and TG2. As a matrix crosslinking enzyme, TG2 mediates the polymerization of osteopontin and appears to function in the aggregation of matrix proteins in agarose microcapsules. Aggregation of polymeric osteopontin may have concentrated integrin ligand binding sites, as well as potentially increasing matrix rigidity, and thereby potentially stimulating prosurvival signalling pathways. Increased survival of MSCs in these microcapsules suggests a pivotal role of the molecular organization and physical properties of the surrounding matrix in modulating cell function. It is anticipated that these matrix supplemented microcapsules will harness the regenerative potential of MSCs and thereby translate to maximum functional recovery of damaged tissue.

6.0 References

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